ADAPTIVE RESPONSES OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS ATCC 4931 BIOFILMS TO NUTRIENT LAMINAR FLOW AND BENZALKONIUM CHLORIDE TREATMENT

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ABSTRACT

Salmonella enterica serovar Enteritidis is an important biofilm-forming food-borne pathogen. This study examined the adaptive responses of *Salmonella* serovar Enteritidis biofilms to different environmental conditions such as flow velocity and benzalkonium chloride (BC) treatment. The influence of a 10-fold difference in nutrient laminar flow velocity on the dynamics of biofilm formation and protein expression profiles was compared. The mode of development and architecture of low-flow and high-flow biofilms were distinct. Exopolymer composition of the two biofilms was also different. However, no major shift in protein expression was seen between the biofilms, nor were there any stress response proteins involved. The biofilms altered their architecture in response to flow, presumably assuming a structure that minimized overall biofilm stress. An empirically-determined shear-inducing flow was applied on high-flow biofilms, fractionating the biofilms into shearable and non-shearable regions. Length:width indices of cells from the two biofilm regions, as well as planktonic cells from biofilm effluent and continuous culture were determined to be 3.2, 2.3, 2.2, and 1.7, respectively. Expression of proteins involved in cold-shock response, adaptation, and broad regulatory functions in the shearable region, and expression of protein involved in heat-shock response and chaperonin function in the non-shearable region indicated that the physiological status of cells in two biofilm regions was also distinct.

The development of biofilm adaptive resistance to BC was then examined. Adapted biofilms survived a lethal BC challenge and re-grew, whereas unadapted biofilms did not. Proteins up-regulated following adaptation included those involved in energy metabolism, amino acid and protein biosynthesis, nutrient-transportation, adaptation, detoxification, and 1,2-propanediol degradation. A putative universal stress protein was also up-regulated. Cold-shock response, stress response, and detoxification are suggested to play roles in adaptive resistance to BC. Functional differences in adaptive response and survival of plankonic and biofilm cells adapted to BC were also studied. The proportion of BC-adapted biofilm cells that survived a lethal BC exposure and heat-shock was significantly higher than that of BC-adapted planktonic cells. Enhanced biofilm-specific up-regulation of various proteins, coupled with alterations in

cell surface roughness and shift in fatty acid composition are proposed to function in the enhanced survival of BC-adapted biofilm cells, relative to BC-adapted planktonic cells.

It is concluded that biofilms adapt to the stress conditions by means of community, cellular, and sub-cellular level responses. These adaptive responses help the biofilms to enhance their ability for survival in the nature, especially those formed in critical environments such as healthcare facilities, the food industry, and households.

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LIST OF ABBREVIATIONS

Å	Angstrom(s)
°C	degree Celsius
µg	Microgram(s)
µm	Micrometer(s)
~	Approximately
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
ACT	After continuous treatment
AFM	Atomic force microscopy / micrograph
ALT	After lethal treatment
ATCC	American type culture collection
BC	Benzalkonium chloride
BCFA	Branched chain fatty acid
ca.	Circa
capLC	Capillary liquid chromatography
CCD	Charge-coupled device
cDNA	Complimentary DNA
CFA	Cyclic fatty acid
cfu	Colony forming unit
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CLSM	Confocal laser scanning microscopy / micrograph
cm	Centimeter(s)
CY5	Cyanine dye
d	Cellular depth
DTT	DL-Dithiothreitol
DFM	Dark-field microscopy / micrograph
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds	Double stranded
EDTA	Ethylene diamine tetraacetic acid

EPS	Extracellular polymeric substances / Exopolymeric substances
ESI	Electrospray ionization
FA	Fatty alcohol
FAME	Fatty acid methyl ester
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
GalNAc	*N*-acetylgalactosamine
GFP/*gfp*	Green fluorescent protein / gene
GlcNAc2	*N*-acetylglucosamine
h	Hour(s)
Hz	Hertz
IEF	Isoelectric focussing
IPG	Immobilized pH gradient
k	Cantilever nominal spring constant
kDa	Kilodalton(s)
kHz	Kilohertz
l	Cellular length
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LSD	Least significant difference
lux	Luciferase gene
LWI	Length:width index
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Milliliter(s)
Mr	Molecular mass
mRNA	Messenger RNA
MS	Mass spectrometry
MSDB	Matrix science database
m/z	Mass-to-charge ratio
N	Newton(s)
NA	Not available

NeuNAc	*N*-acetylneuraminic acid
ND	Not detected
NCBInr	National center for biotechnology information database
NIH	National institutes of health
NK	Not known
nm	Nanometer(s)
nN	Nanonewton(s)
OD	Optical density
OTS	Optical thin section
P	Probability
PD	Propanediol
pH	Hydrogen ion (H^+) concentration
p*I*	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
QAC	Quaternary ammonium compound
QS	Quorum sensing
Q-ToF	Quadrupole time-of-flight mass spectrometer
Re	Reynolds number
RG	Re-growth
rH	Relative humidity
RNA	Ribonucleic acid
RNase	Ribonuclease
RO	Reverse osmosis
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature (21 ± 2ºC)
SA	Cell surface area
SA/V	Cell surface area/Cellular volume
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Second(s)

SEM	Standard error of the mean
SF	Summed feature
SFA	Saturated fatty acid
SPCA	Standard plate count agar
Tn	Transposon
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TSP	Trisodium phosphate
TrEMBL	Tr*anslated* European molecular biology laboratory database
TRITC	Tetramethyl rhodamine isothiocyanate
UFA	Unsaturated fatty acid
V	Cellular volume **or** Volt(s)
vol	Volume
vs.	Versus
w	Cellular width
wt	Weight

1. GENERAL INTRODUCTION

Every year foodborne diseases cause millions of illnesses worldwide (Pang et al., 1995; Humphrey, 2004; World Health Organization, 2007a, b). The association of microbial contamination of food and transmission of diseases has been documented since ancient times. The regulations governing food hygiene can be found in numerous early sources such as the Old Testament, and the writings of Confucius, Hinduism, and Islam. Despite our increased knowledge, foodborne diseases are one of the most prevalent health problems in the contemporary world and an important cause of reduced economic productivity (World Health Organization, 1992; 2007a, b). To ensure a supply of wholesome and safe food to the consumer, it requires the synthesis and systematic application of our knowledge of the microbial ecology of foods and the effects of processing to the practical problem of economically and consistently producing foods which have good keeping qualities and are safe to eat (Adams and Moss, 1995). Accordingly, advanced studies concerning the mode of growth, multiplication, survival, and transmission of foodborne pathogens and food-spoilage organisms on foods and food processing surfaces are important in effective prevention and control of foodborne diseases.

Microorganisms attach to surfaces and grow as a microbial community, both in nature and in food systems. The resulting build-up of microorganisms is called a 'slime' or 'biofilm' (Dunne, 2002; Sauer, 2003). Biofilms form on any submerged surface where bacteria and nutrients are present; bacteria typically colonize solid-liquid interfaces where sufficient nutrients exist for growth. Once attached, bacteria multiply, with newly-formed cells attaching to each other as well as to the surface, forming a complex, and often confluent, community of microorganisms (Geesey et al., 1977; Costerton et al., 1978, 1999; Dunne, 2002; Bhinu, 2005). Biofilms have been associated with a number

of foods and food processing surfaces. Foodborne pathogens gain entry into the food from processing surface biofilms (Stepanović et al., 2003), and subsequently colonize and grow on the surface of food; biofilms thus pose a potent threat to the safety of food by being a source of contamination. Food items are contaminated with undesirable spoilage and pathogenic bacteria from sloughed portions of biofilms. Biofilm formation leads to serious hygienic problems and economic losses due to food spoilage and the presence of foodborne pathogens (Korber et al., 1997; Forsythe, 2000). Therefore, it is of utmost importance that food processing equipment should be cleaned and disinfected regularly and sufficiently with an effective disinfectant of appropriate concentration (Luppens et al., 2002). Generally, an effective cleaning and sanitation program will inhibit biofilm formation. However, the ability of the biofilms to become adapted to sub-lethal concentrations of antimicrobial agents and to eventually become resistant to lethal biocide concentrations is of considerable importance to food safety and public health (Russell, 2003a; Humphrey, 2004).

There are a number of food-spoilage and pathogenic microorganisms, including corynebacteria, streptococci, pseudomonads (Lewis and Gilmour, 1987) and *Listeria monocytogenes* (Toquin et al., 1991) that have been found to be associated with biofilms in dairy and meat (poultry) industries. Similar observations were also made by Le Gros et al. (1986) with respect to *Staphylococcus aureus*. Increased inherent resistance of biofilm (attached) bacteria to sanitizers or antimicrobiobial agents is the major factor affecting plant sanitation and product safety. Frank and Koffi (1990) reported the increased resistance of *L. monocytogenes* in biofilms and Holah et al. (1990) reported that *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *S. aureus* biofilms were 10 to 100 times more resistant to food surface disinfectants than their planktonic (free-living or suspended) counterparts. Food can be contaminated through contact with processing equipment that has microbial biofilms growing on them. However, sufficient information on the mode of colonization of the pathogens on food and food processing surfaces, their tolerance/resistance patterns against various antimicrobial agents used in the food industry, genetic mechanisms of stress response involved in resistance etc. is still lacking.

It has been hypothesized that the cells in different regions of biofilms are physiologically distinct which is suggested to be the reason for enhanced antimicrobial resistance and survival of biofilm cells. Development of different physiological states at different depths of the biofilms might play a role, as others (Lewis, 2001; Parsek and Fuqua, 2004) have suggested, such as depth-dependent growth rates as well as depth-dependent survival following the exposure to antimicrobial agents. Physiologic or phenotypic adaptation resulting in "biocide tolerance" has been attributed to biofilms (Donlan and Costerton, 2002; Sheldon, 2005). Biofilm resistance to biocides results from slow microbial growth rates resulting from nutrient depletion within biofilms, binding of the biocides to biofilms, neutralization or degradation of the biocides, and the expression of biofilm-specific phenotypes (Sheldon, 2005; Szomolay et al., 2005). The biofilm and planktonic states are recognized as distinct phenotypes in the "lifecycle" of bacteria, with significant differences in the physiology and morphology of the cells existing in the two phases. It has been reported that while in the biofilm phenotype, the bacteria display enhanced resistance to antimicrobials, differential gene expression, and increased resistance to phagocytic killing than cells in planktonic state (Parsek and Fuqua, 2004; Cerca et al., 2006). Planktonic and biofilm bacteria of *Escherichia coli*, *P. aeruginosa*, *Pseudomonas pseudomallei*, *S. aureus*, and *Streptococcus sanguis* also have significant differences in their minimum inhibitory concentrations (MICs) of various antibiotics to which they were susceptible (Donlan and Costerton, 2002). Various molecular mechanisms have been reported to play roles in the bacterial adaptive responses to antimicrobial compounds. These molecular adaptive mechanisms act either singly or synergistically to confer resistance; among them, "slow" multiplication resulting in "persisters", the SOS response that blocks cell division during the repair of DNA damage, starvation and dormancy, stringent response, cold-shock response, stress response, detoxification, and altered permeability of the outer membrane due to active efflux of the agent are considered important (Gilbert et al., 1990; Bianchi and Baneyx, 1999; Lewis, 2001; Spoering and Lewis, 2001; Parsek and Fuqua, 2004; Braoudaki and Hilton, 2005; Levin and Rozen, 2006). It has been reported that a single microorganism might have multiple, possibly-interconnected adaptive mechanisms depending on the

nature of the antimicrobial agents (Mah and O'Toole, 2001; Campanac et al., 2002; Szomolay et al., 2005).

Salmonella enterica serovar Enteritidis (*Salmonella* serovar Enteritidis) has emerged as one of the most significant foodborne pathogens during the past three decades (Baumler et al., 2000; Guard-Petter, 2001). It is important that the majority of the strains of this organism can grow on surfaces and interfaces to form biofilms composed of self-secreted exopolysaccharide or exopolymeric material (Solano et al., 2002), including on the food processing and food contact surfaces. Thus, *Salmonella* serovar Enteritidis ATCC 4931 has been used as the model organism in this thesis research. The ecophysiology of *Salmonella* serovar Enteritidis biofilms under normal conditions, and conditions where there is exposure to sub-lethal and lethal concentrations of antimicrobial agents require thorough investigation. Similarly, advanced research is yet to be conducted on the effect of the application of shear forces on the substratum adherence of *Salmonella* serovar Enteritidis biofilms and their architecture. Experiments were conducted to investigate the mechanisms of biofilm resistance to the antimicrobial agent, benzalkonium chloride (BC). As an important surface-active, cationic detergent used in the food industry, BC (a quaternary ammonium compound (QAC)) was used as the antimicrobial compound to study its effects on the biofilms at sub-inhibitory and challenge doses. Health Canada recommends the application of QACs at the concentration of $\geq$ 450 ppm for a period of 10 min to disinfect hard surfaces in food processing plants (Health Canada, 1999). By resolving these questions, an effective control of the foodborne infections and food-spoilage could be achieved and thus avoid the enormous economic losses involved.

This thesis consists of four studies that examined the adaptation of *Salmonella* serovar Enteritidis biofilms to nutrient laminar flow velocity and BC treatment, entitled: (i) Architectural adaptation and protein expression patterns of *Salmonella* serovar Enteritidis biofilms under laminar flow conditions, (ii) Cells in shearable and non-shearable regions of *Salmonella* serovar Enteritidis biofilms are morphologically and physiologically distinct, (iii) Adaptive resistance and differential protein expression of *Salmonella* serovar Enteritidis biofilms exposed to benzalkonium chloride, and (iv)

Differential adaptive response and survival of *Salmonella* serovar Enteritidis planktonic and biofilm cells exposed to benzalkonium chloride.

The research work was based on the following hypotheses and technical objectives:

1.1. Hypotheses

1. Architecture and shearability of *Salmonella* serovar Enteritidis biofilms are influenced by nutrient laminar flow velocity,
2. *Salmonella* serovar Enteritidis cells in different biofilm regions are physiologically distinct,
3. *Salmonella* serovar Enteritidis biofilms become adapted to normally-lethal concentrations of BC through long-term exposure to sub-lethal concentrations of the agent, and
4. Adaptive responses of the planktonic and biofilm cells of *Salmonella* serovar Enteritidis to long-term, sub-lethal exposure to BC are physiologically distinct.

1.2. Technical objectives

1. Develop methods for separating biofilm bacteria into different "physiological" zones,
2. Further develop and employ methods to study the physiological/phenotypic difference of biofilm bacteria by microscopic and proteomic analyses, and
3. Develop methods to determine the adaptive responses of biofilm and planktonic bacteria using microscopic, proteomic, and fatty acid profile analyses.

2. LITERATURE REVIEW

The ultimate goal of food microbiology is to provide an adequate, organoleptically satisfying, wholesome, and safe food supply (Hartman, 2001). The increasing number and severity of food poisoning outbreaks worldwide has significantly increased public awareness about food safety. Public concern of food safety has been raised due to recent well-publicized debates on food irradiation, Bovine Spongiform Encephalopathy (BSE), *Escherichia coli* O157:H7, and genetically modified foods (Forsythe, 2000). Food safety as a significant public health concern is gaining much attention in recent years and Governments all over the world are intensifying their efforts to improve food safety. Foodborne illnesses are described as a widespread and growing public health problem both in developing and developed countries (World Health Organization, 2007a, b).

2.1. History of food microbiology

It was only in the 10th century A.D. that microbiological food poisoning was recognized by civil law; Emperor Leo VI of Byzantium issued a proclamation in 900 A.D. that forbade preparation and consumption of blood sausage because of the association between botulism and blood sausage (Tannahill, 1973). As the underlying causes were unknown, microbiological food poisoning was recurrent, and thus botulism reappeared many times. For example, in 1793, many people were affected and six died in Germany after eating blood sausage. It was believed that the illness was caused by a fatty acid. The disease was made reportable and the product again was regulated (Smith, 1977).

Between 1854 and 1864, Louis Pasteur raised heat preservation methods to a scientific basis. He made many discoveries related to microbiology, most notably

providing experimental proof that certain bacteria were associated with food spoilage and caused specific diseases. Hence, Louis Pasteur became the father of the evolving science, food microbiology. In the meantime, during the 1800s, methods to cultivate microorganisms in pure culture and to associate specific bacteria as the causative agents of specific diseases were developed by Robert Koch, Joseph Lister, and others (Chung et al., 1995). The isolation and study of pure bacterial monocultures in the laboratory remained at the center of food microbiology for the next hundred years (Hartman, 2001).

2.2. Food microbiology

Food microbiology is considered to be one of the most diverse of the areas of study within the discipline of microbiology. The term 'food safety microbiology' has occasionally been used to distinguish the study of food pathogens and spoilage organisms from that of beneficial organisms routinely present or being used in the preparation of foods (International Atomic Energy Agency, 2005). Food microbiology encompasses a wide variety of microorganisms including spoilage, probiotic, fermentative, and pathogenic bacteria, molds, yeasts, viruses, and parasites; a diverse composition of foods; a broad spectrum of environmental factors that influence microbial survival and growth; and a multitude of research approaches that range from very applied studies of survival and growth of foodborne microorganisms to basic studies of the mechanisms of pathogenicity of harmful foodborne microorganisms (Doyle et al., 1997).

2.2.1. Food spoilage

One outcome of food spoilage results from the undesirable growth of microorganisms in food is the production of volatile compounds during their metabolism, which the human nose and mouth detect. During harvesting, processing, and handling operations food might become contaminated with a wide range of microorganisms. If the conditions are favorable, microorganisms will multiply and cause spoilage during the distribution and storage of foods. Spoilage is not only due to growth of microorganisms, but also to the production of end metabolites which result in off odors, gas, and slime (Forsythe, 2000). Berrang et al. (1989) observed that modified

atmosphere storage extended the shelf life of asparagus long enough to allow *L. monocytogenes* to reach higher populations than would have occurred under ambient-air storage. Wells and Butterfield (1999) reported that *Salmonella* serovar Typhimurium grew better on tomatoes, potatoes, and onions in the presence of spoilage molds *Botrytis* spp. or *Rhizopus* spp. than when the molds were absent. Similarly, the growth of *E. coli* O157:H7 is known to occur in bruised areas of apples (Dingman, 2000). So, food microbiologists and technologists should always consider whether the benefits of reduced spoilage outweigh safety concerns (Brackett, 2001).

Gill (1983) reported that, despite variations in expectations, most consumers would agree that gross discoloration, off odors, and the development of slime would constitute spoilage of fresh meat. Newton et al. (1978) and Grau (1986) reported that the majority of bacteria on a dressed red meat carcass originate from the hide. Further transfer of bacteria can occur from the hide removal, from contact with workers' hands or from contact of the hide or fleece with the exposed tissue surface (Grau, 1986).

2.2.2. Food safety

Foodborne illnesses have been defined as 'diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food or water' (World Health Organization, 2007a, b). A number of assessments of the relative significance of hazards associated with food have concluded that microorganisms are of paramount importance; however, unconventional agents such as prions, naturally occurring toxins such as mycotoxins and marine biotoxins, persistant organic pollutants such as dioxins and polychlorinated biphenyls, and heavy metals such as lead and mercury are also of major concern (Adams and Moss, 1995; World Health Organization, 2007a, b). The application of useful microbiological criteria should address the issues like the sensitivity of food products relative to safety and quality, the needs for microbiological standards and/or guidelines, assessment of information necessary for establishment of a criterion if one seems to be indicated, and where criteria should be applied (National Advisory Committee on Microbiological Criteria for Foods, 1993).

Stannard (1997) reported the development and use of microbiological limits for a wide range of products and product groups, as a guidance document for all those

involved in the food and catering industries. Pierson and Smoot (2001) reported the mandatory "zero tolerance" limit for foodborne pathogens like *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* in ready-to-eat-foods, fresh ground beef, etc. The Food Safety and Inspection Service of the U.S. Department of Agriculture (FSIS/USDA) coordinates a pathogen reduction program intended to reduce the level of pathogenic microorganisms in meat and poultry products and as part of this program, microbiological testing of carcasses, ground beef, and poultry for the presence of *Salmonella* spp. is required (United States Department of Agriculture, 1995). Establishments whose production process exceeds the national targets for foodborne pathogens are required to reevaluate their processing controls and, with FSIS/USDA oversight, initiate corrective actions (United States Department of Agriculture, 2002).

The ICMSF (International Commission on Microbiological Specifications of Food) recommended that a series of steps to be taken to manage microbiological hazards for foods intended for international trade (International Commission on Microbiological Specifications of Food, 1997), and these steps include conducting a risk assessment and an assessment of risk management options, establishing Food Safety Objectives (FSO) and confirming that the FSO is achievable by application of Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) (Pierson and Smoot, 2001).

2.3. Microbial biofilms

Microorganisms attach to surfaces and grow as microbial communities, both in nature and in food systems. The resulting build-up of microorganisms is called a 'slime' or 'biofilm' (Dunne, 2002; Sauer, 2003). Biofilms are defined as assemblages of microorganisms and their associated extracellular products at an interface and typically attached to an abiotic or biotic surface (Davey and O'Toole, 2000). Nearly two decades ago, Shapiro proposed the view of bacteria as interactive organisms capable of significant collective activity as a general bacterial trait (Shapiro, 1988, 1998; Davey and O'Toole, 2000). Biofilms are very heterogeneous, containing microcolonies of bacterial cells encased in an extracellular polymeric substance (EPS) matrix and separated from other microcolonies by interstitial voids (water channels) (Caldwell et al., 1992a, 1993;

Lewandowski, 2000; Donlan, 2002). Moreover, it is becoming clear that natural assemblages of bacteria within the biofilm matrix function as a cooperative consortium, in a relatively complex and coordinated manner (Caldwell, 1995; Costerton et al., 1995; Davey and O'Toole, 2000). Bacterial biofilms have also been described as sessile bacterial communities that live attached to each other and to surfaces (Danese et al., 2000). Biofilms are important in environmental, industrial, and clinical contexts (Parsek and Fuqua, 2004). It has been reported by National Institutes of Health that more than 60% of all microbial infections are caused by biofilms (Lewis, 2001). It has also been estimated that biofilms are associated with 65% of nosocomial infections and that treatment of these biofilm-based infections costs more than $1 billion annually (Mah and O'Toole, 2001).

Biofilms are formed by the colonization of bacteria at solid-liquid interfaces where sufficient nutrients exist for growth; once attached, they multiply, with newly formed cells attaching to each other as well as to the surface, forming a highly structured, confluent community of microorganisms (Geesey et al., 1977; Costerton et al., 1978; Caldwell et al., 1992a, 1993; Dunne, 2002; Sauer, 2003). Biofilms are formed in a sequential process, which includes the transportation of microorganisms to the surface, initial microbial attachment, formation of microcolonies, and the development of mature biofilm (Figure 2.3.1) (Marshall, 1985; Van Loosdrecht et al., 1990). Microbiological concerns associated with food processing frequently arise from the recurring contamination of processing plant surfaces by microbial biofilms, and thus they represent a growing concern with regard to spoilage of foods and transmission of foodborne diseases.

While the study of standard planktonic cultures are clearly worthwhile, they provide us with a biased view of microbial life since many bacteria in the natural environment grow aggregated with each other, on solid surfaces, and at gas-liquid interfaces (Parsek and Fuqua, 2004). Similarly, the traditional way of culturing bacteria in liquid medium is instrumental in the study of microbial pathogenesis and enlightening as to some of the amazing facets of microbial physiology; however, pure-culture planktonic growth is rarely how bacteria exist in nature (Davey and O'Toole, 2000).

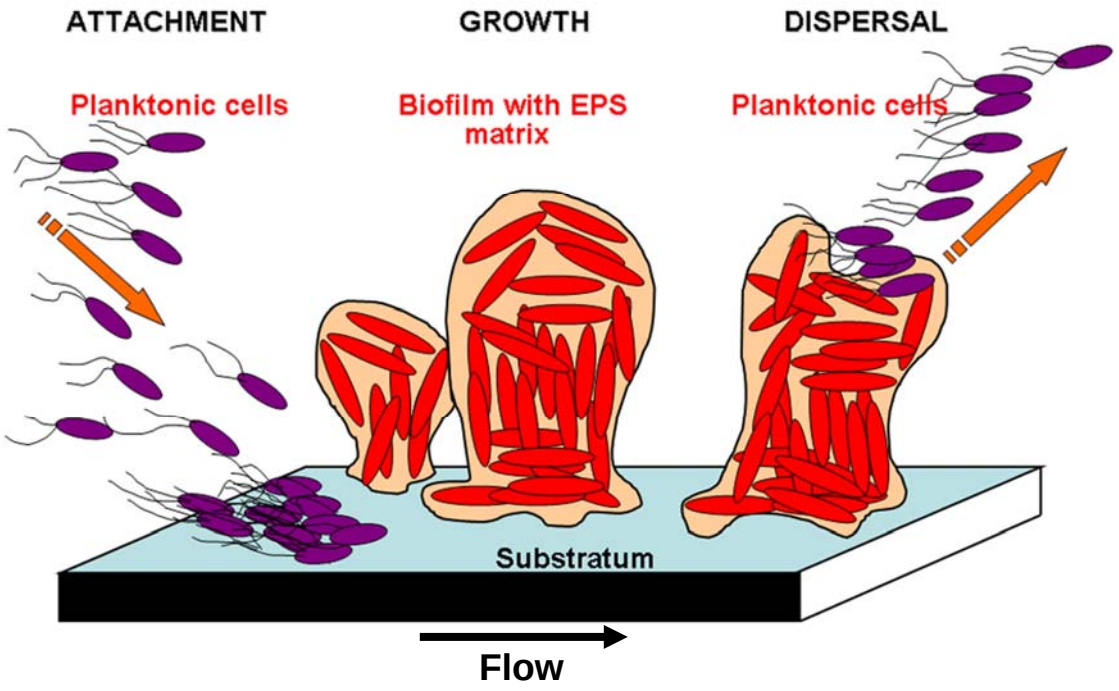

Figure 2.3.1. Stages of biofilm formation. The cells indicated by purple and red shades are planktonic and biofilm cells, respectively.

In natural settings, many bacterial species live predominantly in biofilm communities, with a smaller proportion of the bacterial population subsisting as free-swimming (planktonic) organisms (Danese et al., 2000). Biofilm-associated cells are sessile and differentiated from their suspended counterparts by the generation of an EPS matrix, reduced growth rates, and the up- and down-regulation of specific genes (Sauer, 2003; Bhinu, 2005). The three-dimensional architecture of single-species bacterial biofilms has been described; the two most general features of these biofilms are microcolonies, composed of cells surrounded by large amounts of EPS, and water-filled channels, which have been hypothesized to promote the influx of nutrients and the efflux of waste products (Lawrence et al., 1991; Caldwell et al., 1992a, 1993; Davies et al., 1998; Costerton et al., 1999; Danese et al., 2000). Biofilm formation has been considered as a bacterial developmental process that requires a series of discrete and well-regulated steps (Sauer et al., 2002; O'Toole, 2003). While the exact molecular mechanisms seem to differ from organism to organism, the stages of biofilm development appear to be conserved among a wide range of microbes (O'Toole, 2003).

The application of confocal laser scanning microscopes (CLSM) to biofilm research has fundamentally altered the concept of biofilm structure and composition (Lawrence et al., 1991; Caldwell et al., 1992a, b). CLSM allowed the visualization of fully-hydrated samples of biofilms and revealed elaborate three-dimensional structure (Caldwell et al., 1992a, b, 1993; de Beer et al., 1994; Costerton et al., 1995; de Beer and Stoodley, 1995; Davey and O'Toole, 2000). CLSM has been used very effectively to monitor biofilm development in flow cells. Flow cells are small continuous-flow systems with a viewing port that allows direct observation of the biofilm without disrupting the community (Figure 2.3.2). These systems are once-flow-through, meaning that fresh medium enters the system, passes through the cell, and is collected as waste; the medium is not recycled through the flow cell (Figure 2.3.3) (Lawrence et al., 1997, 2002, 2007; Davey and O'Toole, 2000).

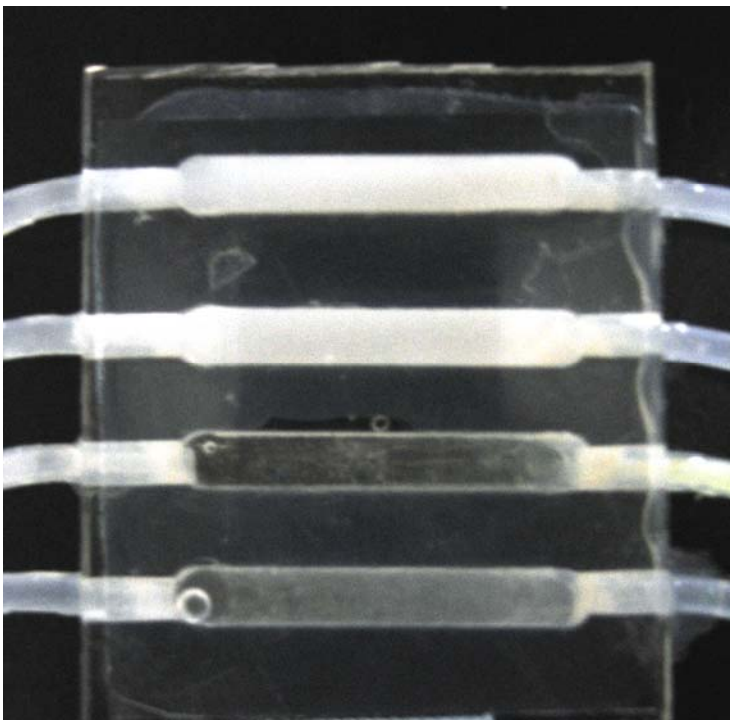

Figure 2.3.2. Image of a flow cell showing the development of bacterial biofilms in the two upper channels.

2.3.1. Composition and structure of microbial biofilms

Although microorganisms can have an independent planktonic existence, an interdependent lifestyle in which they function as an integral part of a community is also possible and is more typical in natural settings; biofilms represent an interdependent community-based existence (Lawrence et al., 1996; Caldwell et al. 1997a, b, c; Davey and O'Toole, 2000; Caldwell et al., 2002). Complex differentiation and collective behavior have been demonstrated for a number of different organisms (e.g., *Bacillus subtilis*, *Serratia liquefaciens*, *Streptomyces coelicolor*, and *Myxococcus* spp.) under a variety of different situations, and these examples testify to the ability of microorganisms to exploit intercellular interactions and communication to facilitate their adaptation to changing environmental parameters (Kaiser and Losick, 1993; Shapiro and Dworkin, 1997; Davey and O'Toole, 2000).

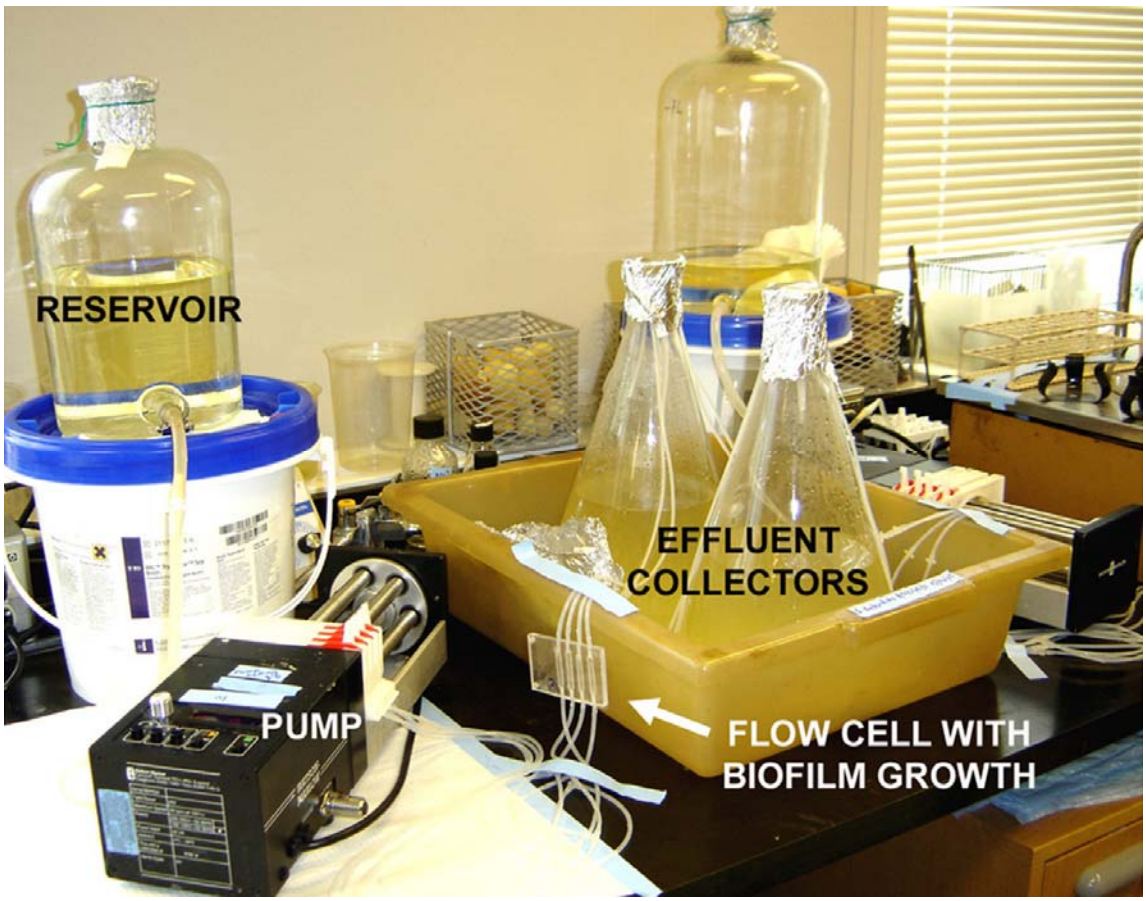

Figure 2.3.3. A continuous nutrient flow system used in biofilm experiments.

The use of advanced microscopic and molecular technologies have shown that biofilms are not simply organism-containing slime layers on surfaces; instead, biofilms represent biological systems with a high level of organization where bacteria form structured, coordinated, and functional communities (O'Toole et al., 2000; Davey and O'Toole, 2000). Caldwell et al. (1997a) have discussed the complex interactions that form the basis of coexistence in these sessile communities. Davey and O'Toole (2000) reported that the microcolonies that constitute biofilm can be composed of single-species populations or multimember communities of bacteria, depending on the environmental parameters under which they are formed. Numerous conditions, such as surface and interface properties, nutrient availability, the composition of the microbial community, and hydrodynamics, can affect biofilm structure (Stoodley et al., 1997, 1999; Davey and O'Toole, 2000). Under high shear stresses, such as on the surface of teeth during chewing, the biofilms (dental plaques) are typically stratified and compacted (Bowden and Li, 1997; Wimpenny and Colasanti, 1997; Davey and O'Toole, 2000). Biofilms have also been examined under various hydrodynamic conditions such as laminar and turbulent flows and it has been shown that biofilm structures become altered in response to flow conditions (Stoodley et al., 1999; Davey and O'Toole, 2000). Biofilms grown under laminar flow conditions were found to be patchy, consisting of rough round cell aggregates separated by interstitial voids; biofilms grown in the turbulent flow conditions were also patchy, but elongated "streamers" that oscillated in the bulk fluid were observed. By observing biofilm development under continuous flow, the effect of perturbations on established biofilms could be ascertained. It was also demonstrated that biofilms were polymorphic and structurally adapted to changes in nutrient availability (Stoodley et al., 1997, 1999; Davey and O'Toole, 2000). Wimpenny and Colasanti (1997) suggested that the substrate concentration is a major factor that determines biofilm structure.

The biofilms formed from single species *in vitro* and those produced in nature by mixed species consortia exhibit similar overall structural features (Costerton et al., 1995; Watnick and Kolter, 1999; Danese et al., 2000; Davey and O'Toole, 2000). It has been noted that every microbial biofilm community is unique although some structural attributes can generally be considered universal (Tolker-Nielsen and Molin, 2000;

Donlan, 2002). Most biofilms have been found to exhibit some level of heterogeneity in that patches of cell aggregates, not monolayers, are interspersed throughout an EPS matrix that varies in density, creating open areas where water channels are formed (Davey and O'Toole, 2000). The interstitial voids or channels are an integral part of the biofilm structure. Water flow through interstitial voids has been demonstrated using particle-tracking techniques (Stoodley et al., 1994). The channels are the lifeline of the system, since they provide a means of circulating nutrients as well as exchanging metabolic/waste products with the bulk fluid layer (Costerton, 1995; Davey and O'Toole, 2000). For example, *in situ* measurements of dissolved O_2 using microelectrodes revealed that O_2 is available in the biofilm as far down as the substrata, indicating that the channels are transporting the oxygenated bulk fluid throughout the biofilm to the surface (Lewandowski et al., 1989, 1993; Davey and O'Toole, 2000; Horn, 2000). Moreover, *in situ* measurements of toluene degradation in a multispecies biofilm indicated that toluene was also available to cells deep within the biofilm, indicating transport through channels (Møller et al., 1996; Davey and O'Toole, 2000). It is construed that the channels are a vital part of the biofilm structure and function, and thus there might be mechanisms for the formation as well as maintenance of channels (Davey and O'Toole, 2000).

2.3.1.1. Exopolymeric substances (EPS)

Biofilms are composed of microbial cells and EPS, and the EPS might account for 50 to 90% of the total organic carbon of biofilms and can be considered as primary matrix material of the biofilm (Flemming et al., 2000; Donlan, 2002). Donlan (2002) reported that EPS may vary in chemical and physical properties, although it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case of the EPS of Gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confers an anionic property (Sutherland, 2001; Donlan, 2002). This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Flemming et al., 2000; Donlan, 2002). In the case of

Gram-positive bacteria, such as the staphylococci, the EPS might be quite different and primarily cationic; the slime of coagulase-negative staphylococci consists of a teichoic acid mixed with small quantities of proteins (Hussain et al., 1993; Donlan, 2002). EPS may also associate with metal ions, divalent cations, and other macromolecules such as proteins, DNA, lipids, and even humic substances (Flemming et al., 2000; Wolfaardt et al., 1999).

There are two important properties of EPS that can have a marked effect on the biofilms; firstly, that the composition and structure of the polysaccharides determine their primary conformation and solubility, and the secondly, that the EPS of biofilms is not generally uniform but varies spatially and temporally (Wolfaardt et al., 1999; Sutherland, 2001; Donlan, 2002). The EPS also vary in its solubility (Donlan, 2002). Lectin-binding specificity of glycoconjugate components of EPS has been used to evaluate bacterial biofilm development by different organisms under different growth conditions (Lawrence et al., 1998; Neu and Lawrence, 1999; Leriche et al., 2000; Neu et al., 2001). Different organisms produce differing amounts of EPS and the amount of EPS increases with the age of the biofilms (Van der Wende and Characklis, 1990). EPS production is known to be affected by the environment, such as nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphorus promote EPS synthesis (Van der Wende and Characklis, 1990; Sutherland, 2001). Slow bacterial growth will also enhance EPS production (Sutherland, 2001). EPS might be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic (Sutherland, 2001; Donlan, 2002). Hydrogen bonding facilitates the EPS to incorporate large amounts of water into its structure, and thus preventing desiccation in some natural biofilms (Donlan, 2002). EPS also contributes to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, probably by direct binding to these agents (Wolfaardt et al., 1999; Donlan, 2000, 2002).

The EPS from bacterial organisms such as *P. aeruginosa*, *E. coli*, and *Salmonella enterica* is comprised mainly of alginate, colanic acid, and cellulose, respectively (Davies et al., 1993; Davies and Geesey, 1995; Prigent-Combaret et al., 1999; Zogaj et al., 2001; Solano et al., 2002). The synthesis of EPS is induced upon attachment of

bacteria to a surface (Danese et al., 2000). There are reports that EPS synthesis is required for the initial attachment to surfaces and for the formation of the complex three-dimensional structure and depth of biofilms formed by various Gram-positive and Gram-negative bacteria (McKenney et al., 1998; Watnick and Kolter, 1999; Danese et al., 2000). Zogaj et al. (2001) reported that cellulose is a component of the matrix produced by biofilm-forming *Salmonella* serovar Typhimurium and identified an operon, named *bcsABZC*, responsible for synthesis of cellulose. Consistent with this finding, Solano et al. (2002) reported that yet another operon, named *bcsEFG*, also responsible for cellulose biosynthesis in both *Salmonella* serovars Enteritidis and Typhimurium. Cellulose deficiency does not affect *Salmonella* serovar Enteritidis virulence but increases the sensitivity to chlorine treatments, strongly suggesting that biofilm formation, in conjunction with cellulose formation, might be important factors in the survival of *Salmonella* serovar Enteritidis on surface environments (Solano et al., 2002).

2.3.2. Biofilms of foodborne pathogens

The foods that we eat carry varied microbial flora, the composition of which depends upon which organisms gain access and how they grow, survive, and interact in food overtime. The microorganisms present will originate from the natural microflora of the raw material and those organisms introduced in the course of harvesting/slaughter, processing, storage, and distribution (Adams and Moss, 1995). Montville and Matthews (2001) stated that most definitions of biofilms reveal that they exist as communities of microbial species embedded in a biopolymer matrix on some substratum. Biofilms are also heterogeneous in time and space, frequently appearing as collections of mushroom-shaped microcolonies with moving water channels between them (Carpentier and Cerf, 1993; Zottola and Sasahara, 1994). Foodborne pathogens like *E. coli* O157:H7, *L. monocytogenes*, *Yersinia enterocolitica*, and *Campylobacter jejuni* form biofilms on food surfaces and food contact equipment, leading to serious health problems and economic losses due to recall of food (Kumar and Anand, 1998).

Biofilm formation is a multistep process in which the substratum first undergoes a conditioning process that allows cells to be adsorbed by weak reversible electrostatic forces. Adhesion via some biopolymer follows subsequently. The synthesis of the matrix

polymer might be upregulated by adsorption of the cell (Montville and Matthews, 2001). The microcolonies have defined boundaries that allow fluid channels to run through the biomatrix. This requires high-level differentiation, quorum sensing, or some kind of cell-to-cell communication to prevent undifferentiated growth from filling in these channels that bring nutrients and remove wastes (Montville and Matthews, 2001). Costerton (1995) reported that the highly-structured biofilm mode of growth provides bacteria with a measure of homoeostasis, a primitive circulatory system, a framework for the development of cooperative and specialized cell functions, and a large measure of protection from antimicrobial agents. Davies et al. (1998) suggested a link between biofilm formation and quorum sensing in *P. aeruginosa*. The use of reporter gene technology can allow one to determine altered gene expression in target bacteria under the range of physicochemical conditions that occur in biofilms (Montville and Matthews, 2001).

Cells in biofilms are more resistant to heat, chemicals, and sanitizers (Scher et al., 2005). This had previously been attributed to the diffusional barrier created by the biomatrix. However, the CLSM images of circulatory channels in hydrated biofilms shatter the barrier hypothesis (Montville and Matthews, 2001). The increased resistance is now attributed, in part, to the very slow growth rate of cells in biofilms (Costerton, 1995). Indeed, cells in the nutrient-depleted interior of the microcolony might be in the viable, but nonculturable (VBNC), state. Leriche and Carpentier (1995) reported that the cells of *Salmonella* serovar Typhimurium following chlorine stress were in VBNC state in single-species biofilms and in binary-species biofilms in association with *Pseudomonas fluorescens*; the presence of *P. fluorescens* resulted in an increased resistance of *Salmonella* serovar Typhimurium to chlorine.

2.3.2.1. Bacterial attachment on meat surfaces

The spoilage of meat generally occurs as a result of the growth of bacteria that have colonized muscle (meat) surfaces and the first stage in colonization and growth involves the attachment of microbial cells to the surface (Benedict, 1988; Dickson and Anderson, 1992). Bacterial attachment to muscle surfaces involves two stages (Firstenberg-Eden, 1981). The first is a loose, reversible sorption that might be related to

van der Waals forces or other physico-chemical factors (Marshall et al., 1971) and the second stage consists of an irreversible attachment to surfaces involving the production of EPS (Costerton et al., 1981). Factors such as cell density, surface characteristics, growth phase, temperature, and motility may also influence the bacterial attachment to the muscle surfaces (Dickson, 1991; Jackson et al., 2001). Differences in the rate of attachment of certain bacterial strains are thought to be a contributing factor in the composition of the initial microbial flora, for example, *Pseudomonas* spp. have been reported to attach more rapidly to meat surfaces than several other types of spoilage bacteria (Firstenberg-Eden et al., 1978; Butler et al., 1979; Jackson et al., 2001).

2.3.2.2. Biofilms on foods and food processing environments

There are a number of pathogenic microorganisms like corynebacteria, streptococci, pseudomonads (Lewis and Gilmour, 1987), and *L. monocytogenes* (Toquin et al., 1991) that have been associated with biofilms in dairy and meat (poultry) industries. Similar observations have also been made by Le Gros et al. (1986) with respect to *S. aureus*. Increased inherent resistance of biofilm bacteria to sanitizers or antimicrobiobial agents is the major factor affecting plant sanitation and product safety. Frank and Koffi (1990) reported the increased resistance of *L. monocytogenes* in biofilms and Holah et al. (1990) reported that *P. aeruginosa*, *S. aureus*, and *P. mirabilis* biofilms were 10 to 100 times more resistant to food surface disinfectants than their planktonic counterparts. Thus, foodborne pathogens growing as biofilms are more important than those growing as planktonic cells in foods.

Biofilm bacteria are embedded in a matrix and are phenotypically different from their planktonic counterparts. The major difference of biofilm organisms is their increased resistance to antimicrobial agents, reportedly 100 or even 1,000 fold more resistant, when compared to the planktonic cells (Gilbert et al., 1990; Forsythe, 2000; Mah and O'Toole, 2001; Korber et al., 2002). Biofilms from natural environments are generally heterogeneous, frequently containing more than one distinct microenvironment, for example, biofilms with aerobic and anaerobic strata are commonly seen. Reviews on biofilms in the food industry emphasize the importance of cleaning prior to sanitation of processing equipment (Carpentier and Cerf, 1993; Zottola

and Sasahara, 1994; Chmielewski and Frank, 2003). Properties of substrates such as hydrophobicity, steric hindrance, surface roughness, and the existence of a "conditioning layer" at the surface, are all thought to be important in the initial bacterial cell attachment process and biofilm development; however, different organisms have diverse patterns of attachment to surfaces, suggesting dissimilar mechanisms of cell attachment (Korber et al., 1993, 1995; Lawrence and Korber, 1994; Lawrence et al., 1995; Cunliffe et al., 1999). Nutrient and water limitation, equipment design, and temperature control are important in biofilm control (Chmielewski and Frank, 2003). Designing of equipment with smooth, highly-polished surfaces prevents biofilm formation by making the initial adhesion step more difficult. Mean reduction values in viable counts of *L. monocytogenes* following treatment with a combination of sodium hypochlorite and heat were approximately 100 times lower in biofilms than for planktonic cells (Frank and Koffi, 1990). Trisodium phosphate (TSP), a post-chill antimicrobial agent for raw poultry, was found to be effective for reducing planktonic and biofilm cell populations of *E. coli* O157:H7, *C. jejuni*, and *Salmonella* serovar Typhimurium; however, there was a significant difference in the elimination of cells based on the species of organism and the phenotype (Somers et al., 1994). The authors found that both planktonic and biofilm cells of *E. coli* O157:H7 were the most sensitive to TSP treatments, followed by *C. jejuni* cells. *L. monocytogenes* cells were the most resistant to the effect of TSP. Biofilm cells of *L. monocytogenes* and *Salmonella* serovar Typhimurium were more resistant to the effect of TSP than the planktonic cells. There are no materials inherently resistant to biofilm formation. Proper cleaning of food contact surfaces ensures that the cells in the nascent biofilms can be reached by sanitizers. Newer methods for control of biofilms include super-high magnetic fields, ultrasound treatment, and high-pulsed electric fields (Kumar and Anand, 1998).

Jessen and Lammert (2003) reported that the bacteria attached to meat processing surfaces could be reduced, but not eradicated, by using one single method or one single detergent or disinfectant. Laboratory experiments and many reports have claimed disinfectants containing peracetic acid to be the most efficient in removing biofilms, whereas chlorine, in practice, appeared more efficient in meat processing plants. Extra disinfection on top of the regular sanitation did not improve the hygienic level, but the

introduction of a scrubbing step reduced the bacterial load and is recommended on critical sites (e.g., control panels and rollers along conveyor belts) and observation sites (e.g., knives and holders for keeping meat products) as well (Jessen and Lammert, 2003).

Previous studies have emphasized the existence of extensive structural complexity and heterogeneity within biofilms (Lewandowski et al., 1989, 1993; Korber et al., 1993; Wolfaardt et al., 1994; Lawrence et al., 1996). The development of heterogeneity in microbial biofilms is a function of a series of factors extrinsic to the bacteria (qualities of the substratum, environmental stresses, nutrient availability, flow rates, and effects of grazing) and also intrinsic (chemotactic and sensory responses, metabolic pathways, and interactions such as aggregation and co-aggregation) (Lawrence et al., 1995). There are numerous adaptive mechanisms employed by microbial communities that facilitate their reproductive success under the broad range of conditions encountered at solid-liquid interfaces, like abundant production of EPS, the chemical nature of which varies with the ambient conditions and the species involved (Møller et al., 1997).

Caldwell et al. (1997b) reported that the laboratory cultivation of microbial consortia and communities is necessary to gain an understanding of the community-level mechanisms for specific environmental processes. Karthikeyan et al. (1999) reported that the chemical nature of the substrate and the method of cultivation had a significant impact on the growth potential and metabolic capability of a microbial community. Karthikeyan et al. (2001) suggested that microbial communities need synergisms (resulting from both internal and external recombination processes) for their optimal growth, just as individuals need molecular and cellular synergisms for their optimal growth.

From a meat-processing perspective, biofilms have the potential to constitute a long term recontamination threat for meat and meat products. So, an understanding of bacterial attachment as well as biofilm formation and persistence is fundamental for the development of antibacterial intervention strategies suitable for the meat industry. Organisms that survive sanitation steps might potentially proliferate and develop into microbial biofilms on meat surfaces before consumption, if optimal conditions are

encountered. The sources for recontamination in the meat industry include water distribution systems, cutting boards, knifes, tables, hooks, conveyors, and employees. So, it is clear that more effective control of food pathogens is dependent upon a better understanding of the factors which lead to the survival of surface and carcass-borne bacteria after antimicrobial treatment. Various antimicrobial agents used in the meat industry as disinfectants are H_2O_2, ozone, chlorine dioxide, acetic acid, lactic acid, and TSP (Korber et al., 1997, 2002; Somers et al., 1994). The membrane-active compounds such as benzalkonium chloride and oxidizing agents such as sodium hypochlorite are also commonly used in the food industry as disinfectants (Luppens et al., 2002). All the stresses to which surface bacteria in the food industry are subjected (chemical stress, desiccation, and starvation) seem to be conducive to enhanced bacterial resistance to disinfection (Leriche and Carpentier, 1995). Bower and Daeschel (1999) reported that foodborne organisms are conferred with resistance by biofilm formation on food processing surfaces as an adaptive response to protect colonies from cleaning and sanitation and that, resistant organisms can also develop as a result of physical processes used in food preservation, such as acid treatments and irradiation processes.

The chances of contamination of food during processing are mostly from surface-borne pathogens as they are so difficult to eradicate from spoilable meat products. Because of their ubiquity in processing plants, biofilms have the potential to constitute a long-term recontamination threat for meat and meat products (Mittelman, 1998; Arnold and Silvers, 2000). Thus, biofilms have been quantified in meat-processing plants (Hood and Zottola, 1997) and attempts have also been made to visualize biofilms on meat tissue surfaces (Delaquis et al., 1992). However, little work has actually been carried out on the formation of biofilms on meat surfaces. Most of the studies were on biofilms formed on model surfaces like flow cells made-up of various hydrophilic (glass and stainless steel) and hydrophobic substances (polystyrene) (Luppens et al., 2002).

2.3.3. Microbial biofilms and antimicrobial resistance

Organisms that survive sanitation steps might potentially proliferate and develop into microbial biofilms on meat surfaces before consumption if the correct conditions are encountered (Korber et al., 1997). Some work has been done on biofilm formation on

meat surfaces (Delaquis et al., 1992; Cabedo et al., 1997). Bacteria living within biofilms are inherently more resistant to antimicrobial treatments than bacteria suspended in solution or grown in batch culture (Ronner and Wong, 1993; Somers et al., 1994; Desai et al., 1998; Norwood and Gilmour, 2000). Possible explanation for increased resistance of biofilm bacteria include limitations to the free diffusion of antimicrobial agents through biofilm matrix (restricted penetration), interaction of the antimicrobial agent with the biofilm matrix, variability in the physical and chemical environments associated with individual biofilm bacteria or regions of biofilm (e.g., varied conditions of pH, osmotic strength, or nutrients), and varied levels of metabolic activity within the biofilm milieu, decreased growth rate, and expression of possible biofilm-specific resistance genes (Brown et al., 1988; Gilbert et al., 1990; Lewis, 2001; Korber et al., 1994, 1997). Anderl et al. (2000) reported the role of limited penetration of ampicillin and ciprofloxacin in *Klebsiella pneumoniae* biofilm resistance. Moreover, several mutations (*hip*, *vncS*, *sulA*, and *mar* genes) can dramatically increase the number of surviving and persisting cells in a population like biofilms, apparently without adversely affecting cell functions (Lewis, 2001).

Mah and O'Toole (2001) reported that owing to the heterogeneous nature of the biofilms, it is likely that multiple resistance mechanisms are at work within a single community, such as slow growth and/or induction of an *rpoS*-mediated stress response, along with the physical and/or chemical structure of EPS or other aspects of biofilm architecture could confer biofilm resistance to biocides. Some of the phenomena that are postulated to contribute to the biofilm defense include expression of biofilm-specific biocide-resistant phenotypes and the recognition of antimicrobial challenge and active deployment of protective stress responses by a subpopulation of the biofilm cells (Stewart and Costerton, 2001; Szomolay et al., 2005).

2.3.4. Adaptive responses to antimicrobial agents in bacterial biofilms

Bacterial biofilms demonstrate adaptive resistance in response to antimicrobial stress more effectively than corresponding planktonic populations (Szomolay et al., 2005). There is mounting evidence that microorganisms in biofilms actively respond to antimicrobial challenges (Szomolay et al., 2005). *P. aeruginosa* biofilms responded to

treatment with imipenem by producing a *β*-lactamase enzyme that deactivates this antibiotic (Giwercman et al., 1991). Similarly, *P. aeruginosa* biofilms increased their capacity to neutralize monochloramine upon exposure to this agent; when biofilms were treated with repeated doses of monochloramine, the second dose was less effective than the first dose even though the biofilm was thinner at the time of the second application (Sanderson and Stewart, 1997). Elkins et al. (1999) used a reporter gene fusion for *katB*, a catalase, to show that this enzyme was induced during treatment of *P. aeruginosa* biofilms with 50 mM H_2O_2. The same H_2O_2 concentration, when applied to planktonic cells, overwhelmed the bacteria and they were not able to exhibit any detectable response. There are also reports that bacteria in biofilms can respond to antibiotic treatment by increasing the synthesis of EPS that contribute to the matrix of the biofilm (Sailer et al., 2003; Bagge et al., 2004).

While biofilms are exposed to antimicrobial agents, reaction-diffusion limited penetration might result in only low levels of the antimicrobial agent reaching the deeper regions of the biofilms (Szomolay et al., 2005). Thus, the sheltered cells are then able to enter an adapted-resistant state if the local time scale for adaptation is faster than that of disinfection, and this mechanism is not available to a planktonic population (Szomolay et al., 2005). The authors illustrated a mathematical model that investigated the potential for an adaptive stress response to contribute to the protection of cells in a biofilm. If an antimicrobial-induced stress response is more effectively deployed in a biofilm, there must be either unique regulation that occurs in the biofilm mode of growth or the conditions in a biofilm must favor induction of the stress response over killing of the cell. The results indicated that for a sufficiently thick biofilm, cells in the biofilm implement adaptive responses more effectively than do planktonic cells (Szomolay et al., 2005). Based on the results of the study, the authors concluded that effective disinfection of the biofilms requires an applied biocide concentration that increases quadratically or exponentially with biofilm thickness (Szomolay et al., 2005).

2.3.5. Genetic basis of biofilm formation

Bacterial structural components involved in initial attachment have been best characterized through mutation analysis, and specific structural components like flagella,

pili, flagella, and adhesins of *Pseudomonas putida* play a critical role in facilitating bacterial interaction with surfaces (Sauer and Camper, 2001). Purevdorj et al. (2002) reported the ability of a cell signaling *lasI* mutant, *P. aeruginosa* PAO1-JP1, to form a biofilm in high shear flow and opined that signaling mechanisms are not required for the formation of strongly-adhered biofilms. Heydron et al. (2002) reported that an *rpoS* mutant of *P. aeruginosa* formed densely-packed biofilms that were significantly thicker than those of the wild-type, where as the *lasI* mutant was indistinguishable from the wild-type at all points. In *E. coli*, attachment is reduced by mutations in the *csgA* gene, a biosynthetic curlin gene (Vidal et al., 1998; Dorel et al., 1999), and in the type I pili biosynthesis gene *fimH*, which encodes a mannose-specific adhesin (Pratt and Kottler, 1998). Similar observations on the loss of adhesive properties due to mutations in pili genes have also been reported in *P. aeruginosa* (O'Toole and Kolter, 1998), *Staphylococcus* spp. (Mack et al., 1994; Heilmann et al., 1997; Rupp et al., 1999), and *Vibrio cholerae* (Watnick et al., 1999). Mutations in surface and membrane proteins like a Calcium-binding protein, a hemolysin, a peptide transporter and a potential glutathione-regulated K^+ efflux pump caused defects in attachment of *P. putida* to corn seeds (Espinosa-Urgel et al., 2000). Reduced attachment to biotic and abiotic surfaces was observed in an O-polysaccharide-deficient *Pseudomonas* spp. (Dekkers et al., 1998; DeFlaun et al., 1999). The extracellular polysaccharide alginate was required for the formation of thick, 3-D biofilms of *P. aeruginosa* and was shown to be the intercellular material of *P. aeruginosa* microcolonies (Nivens et al., 2001). Similar observations were made with *E. coli* strains with mutations in the lipopolysaccharide core biosynthesis genes *rfaG*, *rfaP*, and *galU* (Genevaux et al., 1999; Rodriguez-Herva et al., 1999). Attachment to surfaces is thought to initiate a cascade of changes in the bacterial cells. In *E. coli*, up-regulation after attachment was observed for OmpC, the *proU* operon, colanic acid exopolysccharide production, tripeptidaseT, and the nickel high affinity transport system (*nikA*) (Prigent-Combaret et al., 1999).

2.3.6. Quorum sensing in biofilms

Recent studies have linked quorum sensing and biofilm formation. Quorum sensing is a broadly conserved virulence mechanism among bacterial pathogens of plants

and animals (Guard-Petter, 2001; Withers et al., 2001). The expression of various proteins and virulence factors by the cells in biofilms is under the control of quorum sensing (Fuqua and Greenberg, 1998; Whitley et al., 1999). Maturation and differentiation into microcolonies were found to be dependent on the signal molecule *N*-3-(oxooctanoyl)-L-homoserine lactone ($3OC_{12}$-HSL) and this finding led to the speculation that cell-to-cell signaling induced by the high density of bacteria within biofilms might play a role in the establishment of a biofilm-specific physiological state (Davies et al., 1998). Sauer and Camper (2001) suggested that in the case of early biofilm development, quorum sensing does not regulate the changes in the protein patterns and gene expression patterns, and therefore is not responsible for the observed change in phenotype in *P. putida*. Biofilm formation, biofilm architecture, and sloughing in *Serratia marcescens* have been reported to be controlled by quorum sensing (Rice et al., 2005). Under high-nutrient conditions, an *N*-acyl homoserine lactone quorum-sensing mutant formed biofilms indistinguishable from the wild-type biofilms. Likewise, other quorum-sensing-dependant behaviors, such as swarming motility, could be rendered quorum-sensing-independent by manipulating the growth medium. Quorum sensing was found to be involved in the sloughing of the filamentous biofilm formed by wild-type *S. marcescens* (Rice et al., 2005). However, the mechanisms of quorum sensing of foodborne pathogens require more thorough investigation.

2.3.7. Protein expression in biofilms

Alginate has been implicated as the embedding matrix in biofilms of *P. aeruginosa* and the adherence of pseudomonads to a solid surface up-regulates the expression of the alginate biosynthetic genes *algC* (Davies et al., 1993; Davies and Geesey, 1995) and *algD* (Hoyle et al., 1993). The regulation of alginate is mediated by a hierarchy of proteins, including those encoded by the *algTmucABCD* operon. The presence and composition of lipopolysaccharides that affect the electrostatic interactions between bacteria and substratum contribute to the adhesiveness of *Pseudomonas* spp. (Williams and Fletcher, 1996). Sauer and Camper's (2001) report using 2D-PAGE analysis of protein preparations of *P. putida* revealed 15 genes that underwent enhanced expression and 30 genes that underwent reduced expression during bacterial adhesion.

Some of the down-regulated proteins included a protein with homology to the ABC transporter, PotF; an outer membrane protein, NlpD; and five proteins that were involved in amino acid metabolism. Subtractive cDNA hybridization of mRNA revealed 40 genes that were differentially expressed following initial attachment of *P. putida*. Sternberg et al. (1999) reported that individual organisms in biofilms displayed heterogeneous behavior with respect to their metabolic activity, growth status, and gene expression pattern. The authors used a reporter system, carrying fusions between the growth-regulated *E. coli rrnB*P1 promoter and different variant *gfp* genes for growth-activity monitoring, by measuring the rates of rRNA synthesis. De Kievit et al. (2001b) reported that during the course of biofilm development of *P. aeruginosa*, *lasI* gene expression was found to progressively decrease over time, whereas *rhlI* expression remained steady throughout biofilm development. Spatial analysis revealed that *lasI* and *rhlI* were maximally expressed in cells located at the substratum and decreasingly expressed with increasing biofilm height. These observations indicate that different biofilm regions have possibly distinct physiology.

2.4. Analyses of biofilms

Two successful approaches for probing biofilm structure, composition, and function include the use of microsensors (a physical method) or the use of reporter genes (a biological method) such as green fluorescent protein gene (*gfp*) to tag genes in order to acquire spatial information using direct microscopic investigation (Neu, 2000).

2.4.1. Biosensors in biofilm analyses

Early microelectrode studies in biofilms were published by Bungay et al. (1969). Kuenen et al. (1986) reported the O_2 profiles of trickling filter biofilms and their research focused on light-dark effects and flow velocity on the O_2 concentration in the biofilm. The diffusion coefficient for glucose could be determined in a biocatalyst gel bead by measuring glucose profiles with a glucose enzyme microelectrode (Cronenberg and Van den Heuvel, 1991). Horn (2000) reported that microelectrodes could be used for purposes of studying substrate conversion and transport in the growing biofilm. In a biofilm tube reactor, flow velocity, substrate, and O_2 conditions could be controlled

independently (Horn, 2000), and the biofilm thickness could be measured after draining the whole reactor for 30 minutes (Characklis, 1989). The Clark-type O_2 microelectrode, a sensitive and useful tool in biofilm research combined with CLSM for studying the structure, is suggested to remain the most important tool in biofilm research (Horn, 2000). Stewart et al. (2000) studied the penetration of H_2O_2 into biofilms formed by wild-type and catalase-deficient *P. aeruginosa* using microelectrodes. Overall, there are not many reports on microelectrode studies of biofilms of foodborne pathogens.

2.4.2. Confocal laser scanning microscopy (CLSM)

Various microscopic techniques, namely, normal light microcopy, transmission electron microcopy, scanning electron microscopy, atomic force microscopy, and confocal laser scanning microscopy, have been used for the study of biofilms (Neu, 2000). CLSM has been widely used to study biofilm structure and function (Lawrence et al., 1991; Caldwell et al., 1992a, b). Before the study by Lawrence et al. (1991), electron microscopy (EM) was the method of choice to examine microbial biofilms under high resolution (Davey and O'Toole, 2000). Compared to EM, CLSM allows the visualization of fully hydrated samples and reveals the elaborate 3-D structure of the biofilm (Lawrence et al., 1991; Costerton et al., 1995; de Beer et al., 1994; de Beer and Stoodley, 1995). CLSM systems might be run in either the reflection mode, transmission (laser) mode or using labels such as FITC, TRITC, and CY5 in the most commonly-used fluorescence mode, using fluorescent probes such as DAPI, SNARF, BAPTA, Mag-Fura, and numerous types of gene probes for phylogenetic and structural genes (Neu, 2000). The diversity and spatial distribution of a population within a community can be studied by identifying cells hybridized *in situ* with fluorescence rRNA-targeted probes (Amann et al., 1996).

Studies combining fluorescent *in situ* hybridization (FISH) with microelectrode analysis for detecting pH, O_2, or sulfide profiles have been performed to evaluate the distribution of different populations in relationship to chemical profiles (Harmsen et al., 1996). Differential expression of genes in a population can be monitored by FISH and by PCR, with primers specific for the genes along with CLSM (Wagner et al., 1998). Using hybridization with fluorescent probes or by staining with Acridine Orange, the growth

rate of the biofilm cells can be evaluated by detecting the cellular ribosome (rRNA) content (Davey and O'Toole, 2000). FISH can be combined with specific enzyme activity probes (e.g., phosphatase activity) so as to assign functions of certain phylogenetic groups (Van Ommen Kloeke and Geesey, 1999). CLSM and its variations including *xy/xz* and *xt*-dimension imaging, reflection mode, simultaneous multichannel scanning, fluorescence recovery after photobleaching (FRAP), photoactivation of fluorescence (PAF), color spectroscopy and Raman spectroscopy, can be effectively utilized in future studies of biofilms (Neu, 2000).

2.4.3. Atomic force microscopy (AFM)

Atomic force microscope (AFM) or scanning force microscope was invented by Binnig, Quate, and Gerber in 1986. AFM has extended the use of scanning probe microscopes to the observation of nonconductive surfaces, and that has opened up unexpected possibilities for the surface analysis of bacterial specimens (Figure 2.4.1) (Braga and Ricci, 1998). AFM has been extensively used in the past decade to probe structural and physical properties of microbial surfaces, indicating that the instrument is taking root in the microbiological science community (Dufrêne, 2002, 2003, 2004). This technique is able to provide new information on bacterial cell surfaces by allowing structural changes to be revealed directly in growth medium (Dufrêne, 2004). Cross et al. (2006) reported that AFM could also be used to investigate function and cellular nanomechanical characterization of local cellular properties inherent to specific biological cells. AFM is a versatile technique that can use probes of different properties and features, either in contact or tapping mode, depending on the specificity of the objective under investigation. Moreover, AFM may be used to study biological surfaces on a real-time basis in different environments, gaseous or aqueous, with very little surface preparation and no surface coating (Del Sol et al., 2007).

Braga and Ricci (1998) reported that AFM could be efficiently used for investigating the morphological and surface alterations of *E. coli* at an unprecedented degree of resolution subsequent to treatment with sub-MICs and supra-MICs of a *β*-lactam antibiotic, cefodizime. Touhami et al. (2004) investigated the growth and division of *S. aureus* by AFM and thin-section transmission electron microscopy and found that

there was good correlation of the structural events of division using the two microscopies. AFM was able to provide additional information with respect to surface features such as murosomes, concentric surface rings and central depression, and the formation of new cell walls following cell separation.

Dittrich and Obst (2004) reported that AFM could be efficiently used for studying calcite crystal growth on the cell surface of picocyanobacteria. Cross et al. (2006) reported the application of AFM to study the structure-function relationships of biofilm-forming *Streptococcus mutans* with respect to mutations in specific genes (*gtfB*, *gtfC*, and *gtfD*) that encode surface proteins. AFM was also used to characterize changes to the cell surface of *S. coelicolor* during the life cycle and showed how chaplin (ChpA to ChpH) and rodlin (RdlA and RdlB) proteins contributed to the formation of hyphal fibrous layers of differing stabilities (Del Sol et al., 2007).

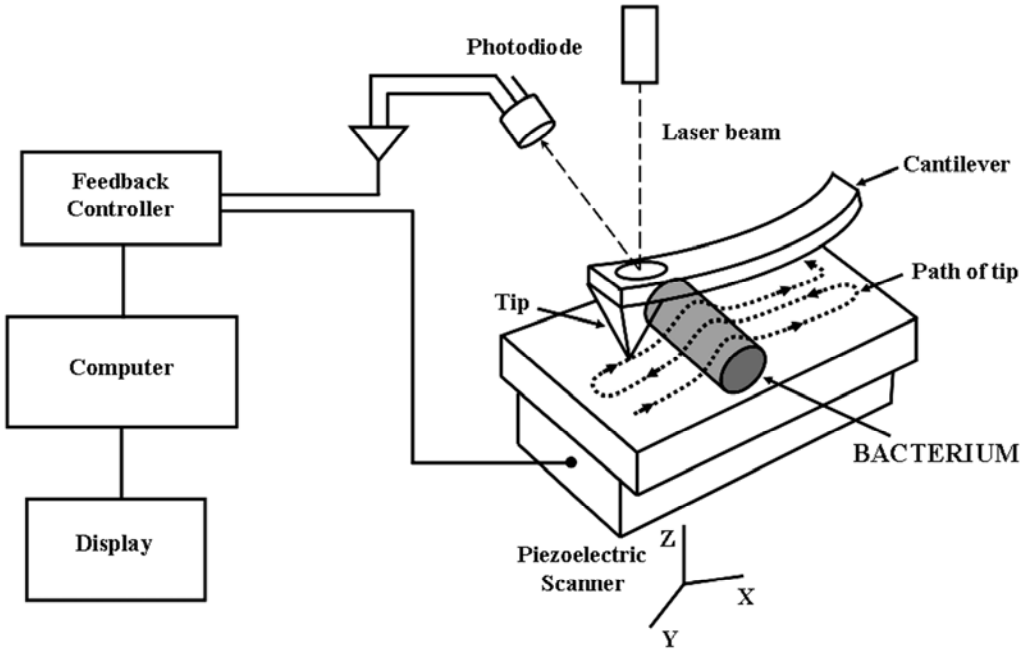

Figure 2.4.1. Schematic diagram showing atomic force microscopy of a bacterial cell [adapted with permission from Braga and Ricci (1998)].

Ahimou et al. (2007a, b) reported the application of AFM for measuring biofilm cohesive strength per unit volume in different biofilm locations of mixed-culture biofilms, finding that cohesive energy increased with biofilm depth and calcium

incorporation. In conjunction with AFM, it has been concluded that biofilm cohesive strength was directly proportional to biofilm EPS concentration and dissolved oxygen concentration; however, biofilm cohesion was found to be unaffected by protein concentration (Ahimou et al., 2007a).

2.4.4. Green fluorescent protein-based reporter systems

Green fluorescent protein (GFP)-based reporter systems have been developed for biofilm-forming Gram-positive (*Streptococcus gordonii*, *L. monocytogenes*, and *S. aureus*) and Gram-negative (*Pseudomonas* spp.) bacteria (Surez et al., 1997; Hansen et al., 2001; Qazi et al., 2001) and used to facilitate CLSM analyses. Cho and Kim (1999a, b) reported the development of a *Salmonella* serovar Typhi strain, chromosomally marked with either the *gfp*, or the *lux* (luciferase) gene, using hybrid transposon mini-Tn5 transconjugated from *E. coli* to *Salmonella* serovar Typhi, for verifying a viable but nonculturable state of bacteria in the environment. Sternberg et al. (1999) used a novel reporter system comprised of a transposon cassette-carrying fusions between the growth-regulated *E. coli rrnB*P1 promoter and different variant *gfp* genes for the monitoring of growth-activity of *P. putida* by measuring the rates of rRNA synthesis. De Kievit et al. (2001b) reported the contributions of QS genes *lasI* and *rhlI* to biofilm maturation in *P. aeruginosa*. In this case, the authors utilized the fusions of these genes to unstable GFP in concert with CLSM and performed a real-time temporal and spatial study of the genes in a flowing environment.

2.4.5. Proteomic analyses of biofilms

Protein profiles are usually obtained by polyacrylamide gel electrophoresis, agarose gel electrophoresis, or by chromatography. Two dimensional gel electrophoresis for protein analysis of bacteria are typically conducted according to the principles of O'Farrell (1975) as modified by Görg et al. (2000). The phenotypic changes that occur during *P. putida* biofilm formation were studied by proteomic analysis of whole-cell extracts and by cDNA subtractive hybridization of mRNA (Sauer and Camper, 2001). Proteomic studies carried out using 2D-PAGE of the whole crude protein extracts of planktonic and sessile cells revealed 45 differences in the protein profile (Sauer and

Camper, 2001). Proteomic analyses of important bacterial pathogens in biofilms have been reported recently, and it was found that there were a number of proteins that are up-regulated while biofilms are being formed and subsequently matured (Sauer et al., 2002; Resch et al., 2006). Trémoulet et al. (2002) compared the proteomes of *E. coli* O157:H7 cultivated in planktonic and biofilm phenotypes and found 14 proteins were up-regulated and three proteins were down-regulated in the biofilms. The differentially-expressed proteins were those involved in general metabolism, sugar and amino acid transportation, and regulatory functions.

Hefford et al. (2005) reported that proteomic analyses of *L. monocytogenes* biofilms revealed that proteins up-regulated in biofilm-grown cells were those involved in stress response, envelope and protein synthesis, biosynthesis, energy generation, and regulatory functions. In *S. aureus*, the proteomic analyses using 2D-PAGE and ESI-MS/MS revealed that biofilm cells expressed higher levels of proteins associated with cell attachment and peptidoglycan synthesis, and in particular, fibrinogen-binding proteins (Resch et al., 2006). Moreover, biofilm cells expressed more staphylococcal accessory regulator A protein (SarA), which corroborates the positive effect of SarA on the expression of the intracellular adhesion operon *ica* and biofilm growth. Recently, Mikkelsen et al. (2007) used proteomic analyses to investigate the interrelationships between colonies, biofilms, and planktonic cells of *P. aeruginosa* under comparable conditions. The authors concluded that protein profiles of cells in colonies resembled those of planktonic cells; furthermore, the protein profiles of biofilms were found to more closely resemble those of exponentially growing planktonic cells than those of planktonic cells in the stationary phase. Recent advances in 2D-PAGE and mass spectrometry analyses, such as fluorescence 2D difference gel electrophoresis (DIGE) and surface-enhanced laser desorption ionization-time of flight (SELDI-ToF), respectively, have proven that proteomics is a powerful and promising tool for elucidating intricate cellular processes in bacteria (Merchant and Weinberger, 2000; Tonge et al., 2001).

2.4.6. Fatty acid profile analyses

Fatty acids are a major component of both lipid bilayers (inner and outer membranes) of the cell envelope in Gram-negative bacteria (Doerrler, 2006). Growth temperature, pH, and chemical composition of the microenvironment significantly influence the fatty acid composition in bacteria (Annous et al., 1997; Brown et al., 1997; Sampathkumar et al., 2004). The proper physical state of biological membrane lipids is required for optimal membrane structure and function (Annous et al., 1997). Temperature markedly affects membrane lipid composition, and changes in lipid composition are known to occur in order to maintain an appropriate amount of the liquid-crystalline state (Annous et al., 1997). The major way in which bacteria, which generally lack cholesterol, maintain this ideal membrane physical state is by changing their fatty acid composition (Suutari and Laakso, 1994; Annous et al., 1997). As the growth temperature decreases, lower-melting-point fatty acids are incorporated into lipids, which have lower phase transition temperatures and *vice versa*. This has been described as a homeoviscous adaptation, where the ideal membrane lipid physical state is maintained through lipid changes in response to changes in growth temperature (Sinensky, 1974; Annous et al., 1997). For example, Suutari and Laakso (1994) have described three modes of adaptation in fatty acid composition in response to temperature: (i) the type of branching in the methyl end of fatty acid, (ii) fatty acid chain length, and (iii) the degree of fatty acid unsaturation. Annous et al. (1997) reported that in *L. monocytogenes*, the fatty acid composition dominated by anteiso-$C_{15:0}$ fatty acid aided survival in cold temperatures by maintaining a fluid, liquid-crystalline state of the membrane lipids.

In *E. coli*, it has been suggested that the acid tolerance at pH 3.0 was correlated with membrane cyclopropane fatty acid (17:0 cyc and 19:0 cyc) content, and thus it was postulated that increased levels of cyclopropane fatty acids might enhance the survival of cells exposed to low pH (Brown et al., 1997). Sampathkumar et al. (2004) reported that alkaline pH- or TSP-pretreatment of *Salmonella* serovar Enteritidis ATCC 4931 cells resulted in a shift in fatty acid composition towards a higher saturated and cyclic to unsaturated fatty acid ratio than seen in the untreated control cells.

2.5. Antimicrobial agents

Antimicrobial agents are a variety of chemical or biological compounds able to inhibit or kill microbes such as viruses, bacteria, and fungi. Antibiotics are used as chemotherapeutic drugs to treat bacterial or fungal infections in human beings and animals (Russell, 2003a). The term biocide includes disinfectants, antiseptics, and preservatives. It does not include antibiotics which, in spite of being biocides in the strictest sense, tend to be categorized separately (Fraise, 2002). Biocides are used for a range of purposes, usually for treatment of inanimate objects such as food processing surfaces (hard surface disinfectants), externally on the skin (antiseptics and topical antimicrobials) to prevent or limit microbial infection, for preoperative skin disinfection, or incorporated as preservatives into pharmaceutical, cosmetic, or other types of products to prevent microbial contamination (McDonnell and Russell, 1999; Russell, 2003a). Agents such as chlorhexidine salts and QACs are used as antiseptics, disinfectants, and preservatives; whereas, others (gluteraldehyde and orthophthalaldehyde) are used predominantly for the disinfection of endospores (Russell, 2003a). Several factors influencing antimicrobial efficacy include concentration, period of contact, pH, temperature, the presence of organic matter or other interfering or enhancing materials or compounds, and the nature, numbers, location, and condition of the microorganisms (Russell, 2003a).

2.5.1. Mechanisms of action

Various antimicrobial agents have unique mechanisms of action on bacterial cells depending on their chemical nature. McDonnell and Russell (1999) reported that different mechanisms of action of antimicrobial agents include destabilization and damage to the cell envelope (cell wall and outer membrane) and cytoplasmic (inner) membrane (e.g., EDTA, gluteraldehyde, and chlorhexidine), cross-linking of macromolecules such as proteins, RNA, and DNA (e.g., formaldehyde and gluteraldehyde), interaction with thiol groups (e.g., silver compounds), DNA intercalation (e.g., acridines), breakage of DNA strands and inhibition of DNA synthesis (e.g., halogens and H_2O_2), and oxidation of thiol groups in enzymes and proteins (e.g., halogens and peroxygens). It was reported that QACs exert their antimicrobial action

through generalized membrane damage involving phospholipid bilayers of the cytoplasmic membrane (McDonnell and Russell, 1999).

2.5.2. Antimicrobial resistance

The first report of biocide resistance was the resistance to chlorine recognized in *Salmonella* serovar Typhi almost 70 years ago (Heathman et al., 1936). Antibiotic resistance was identified shortly after penicillin was used in medical settings; however, links between biocide and antibiotic resistance were recognized only recently (Fraise, 2002). Adaptive resistance as an important aspect of microbial resistance to antimicrobial agents and the cross-resistance (co-resistance) of adapted organisms to related or unrelated biocides/antibiotics has been reported in many bacterial organisms, including some serovars of *S. enterica* (Loughlin et al., 2002; Braoudaki and Hilton, 2004, 2005; Szomolay et al., 2005; Mangalappalli-Illathu and Korber, 2006). Single microorganisms might have multiple, possibly interconnected responses depending on the nature of the antimicrobial agent (Szomolay et al., 2005). For example, *P. aeruginosa* responded to H_2O_2 by inducing catalase (Elkins et al., 1999), to imipenem by inducing *β*-lactamase and alginate synthesis (Bagge et al., 2004), and to tobramycin with a distinct genetic response (Whiteley et al., 2001).

2.5.2.1. Mechanisms of antimicrobial resistance

Increased antibiotic resistance associated with biofilm bacteria has been attributed to antibiotic-modifying enzymes and multidrug efflux pumps (Sauer and Camper, 2001). The authors observed that the antibiotic resistance and virulence factor genes were differentially regulated following initial adhesion, and suggested that this might include a component of the antibiotic efflux system, *mexB*; a streptomycin *str* resistance gene; *β*-lactamase *ampC*; and chitinase *chiA*, and all four gene products were described to be surface induced (Giwercman et al., 1991; Bagge et al., 2000; Baty et al., 2000; Espinosa-Urgel et al., 2000). Changes in gene expression following attachment of organisms has also been described for antibiotic resistance, including *β*-lactamase activity in *P. aeruginosa* (Giwercman et al., 1991), and for antibiotic production such as phenazine synthesis in *Pseudomonas aureofaciens* (Wood et al., 1997). There are reports

that multidrug efflux pumps, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY significantly contribute to antimicrobial resistance in *P. aeruginosa* (De Kievit et al., 2001a; Hocquet et al., 2003). Owing to the links between biocide resistance and antibiotic resistance, there is a real risk that widespread biocide use could exacerbate the already worrying trend of increased antimicrobial resistance in clinically-relevant organisms (Fraise, 2002).

2.5.2.2. Evaluation of antimicrobial resistance

It remains unclear how well most current antimicrobial compounds perform under industrial conditions, and only recently were biofilms actually used in antimicrobial testing. Several techniques have been described for antimicrobial testing with biofilms. Very often batch systems are used for biofilm formation, but a disadvantage of these batch methods is that since little or no shear force is applied, the cells are very loosely attached to the surface and thus are not representative of biofilms in practice (Luppens et al., 2002). The antibacterial activity of TSP in conjunction with plasmolysis-deplasmolysis (PDP), on biofilms of various meat spoilage and pathogenic organisms like *Salmonella* serovar Enteritidis, *E. coli*, *L. monocytogenes*, *Bronchothrix thermosphacta*, and *Pseudomonas* spp. in flow cells and adipose tissue models were evaluated (Korber et al., 1997, 2002). After the extended time courses, when the TSP stress was relieved, biofilms continued to grow within crevices but not in systems without crevices. On pork adipose tissue, higher TSP concentrations were necessary to produce significant decreases in the number of viable cells and that the PDP process generally failed to enhance TSP efficacy. This might be due to the mode of TSP antimicrobial activity, alkaline pH stress, and the chemically complex, buffered nature of meats. TSP was effective in removing attached *E. coli* O157:H7 and *Salmonella* spp. (Kim and Slavic, 1994). Research has shown that TSP can facilitate significant reductions in meat-borne spoilage (Dorsa et al., 1998) and pathogenic organisms (Dickson et al., 1994; Cutter and Rivera-Betancourt, 2000).

Stewart et al. (2000) studied the penetration of H_2O_2 into biofilms formed by wild-type and catalase-deficient *P. aeruginosa* using microelectrodes, and found that the *katA* gene-product, catalase, protected aggregated bacteria by preventing full penetration

of H_2O_2 into biofilms. Luppens et al. (2002) developed a standardized disinfectant test for cells in biofilms using *S. aureus* ATCC 6538 as the test organism, and the membrane-active compound benzalkonium chloride and the oxidizing agent hypochlorite, as disinfectants. Their observation was that the biofilm cells were less susceptible to disinfectants than suspension test cells.

Greer and Dilts (1995) reported the antibacterial effects of lactic acid on the aerobic growth of meat-borne psychrotrophic pathogens (*L. monocytogenes*, *Y. enterocolitica*, and *Aeromonas hydrophila*) and spoilage bacteria (*Pseudomonas fragi* and *B. thermosphacta*) inoculated onto pork fat and lean tissues, and found that bacteria on fat tissue were more sensitive to lactic acid than those associated with lean pork tissue. Even today, reports on the efficient testing methods of the action of disinfectants on biofilms on foods and in the food industry are lacking.

2.5.3. Quaternary ammonium compounds (QACs)

QACs are surface-active agents (surfactants) that have two regions in their molecular structures; one, a hydrocarbon-containing hydrophobic group and the other, a hydrophilic or polar group (McDonnell and Russell, 1999). Depending on the basis of the charge or absence of ionization of the hydrophilic group, surfactants are classified into cationic, anionic, nonionic, and ampholytic (amphoteric) compounds (McDonnell and Russell, 1999). Among them, the cationic agents, as exemplified by QACs, are the most useful antiseptics and disinfectants (Frier, 1971). They are sometimes known as cationic surfactants/detergents because they reduce surface tension at interfaces, and are attracted to negatively-charged particles (i.e., microorganisms). QACs have been used for a variety of clinical purposes (e.g., preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces). In addition to having antimicrobial properties, QACs are also excellent for hard-surface cleaning and deodorization (McDonnell and Russell, 1999).

It is known that QACs are membrane-active agents (Hugo and Frier, 1969) (i.e., with a target site predominantly at the cytoplasmic membrane in bacteria, or the plasma membrane in yeasts) (McDonnell and Russell, 1999). Salton (1968) proposed the following sequence of events occur after microorganisms are exposed to cationic agents:

(i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (Denyer, 1995; McDonnell and Russell, 1999).

The structural functionality of QACs, especially the role of chain length on activity against different bacteria, has been reported previously (Tomlinson et al., 1977; Ioannou et al., 2007). It has been reported that QACs with a C_{16} hydrophobic tail length affected the outer membrane of Gram-negative bacteria more extensively than shorter-chain compounds, possibly due to stronger interaction of C_{16} chain with fatty acid portion of lipid A (Ahlström et al., 1999). A common feature of QACs is their ability to cause membrane damage and cell leakage, primarily due to their adsorption to the bacterial membrane in large amounts (Hamilton, 1968). Monoalkyl QACs bind via ionic and hydrophobic interactions to microbial membrane surfaces, with the cationic head group facing outwards and the hydrophobic tails inserted into the lipid bilayer, causing rearrangement of the membrane and subsequent leakage of intracellular constituents (Ioannou et al., 2007). Ioannou et al. (2007) also reported that generally QACs are initiators of autolysis at low biocide concentrations (9 to 18 μg ml^{-1}), which, together with bactericidal activity, contribute to cell death.

2.5.3.1. Benzalkonium chloride (BC)

BC is a synthetic derivative of ammonium chloride (NH_4Cl); it is a second generation, substituted QAC with high biocidal activity. These synthetic compounds are derived from NH_4Cl with the hydrogen atoms being replaced by organic groups such as methyl, ethyl, and/or benzyl groups. The chemical name of BC is alkyl dimethyl benzyl ammonium chloride (Health Canada, 1999; Ioannou et al., 2007). The chemical structure of BC is comprised of cationic (hydrophobic hydrocarbon structure) and anionic (hydrophilic chloride radical) groups (Figure 2.5.1).

The appearance of methicillin-resistant *S. aureus* (MRSA), a major nosocomial agent which tends to be cross-resistant to BC, a disinfectant widely used in hospitals, has been reported (Al-Masaudi et al., 1988). The increase in resistance of MRSA to *β*-lactam antibiotics, including cefmetazole, cloxacillin, flomoxef, moxalactam, and oxacillin, has been suggested to be due to gene mutations (affecting the efficiency of uptake, activating an efflux pump, or encoding elements regulating the expression of methicillin resistance (e.g., *femA*)) conferring resistance to BC and benzethonium chloride, another cationic detergent (Akimitsu et al., 1999). The *E. coli* MdfA (multidrug transporter) protein was recently identified and shown to confer greater tolerance to both antibiotics and BC (Edgar and Bibi, 1997; McDonnell and Russell, 1999).

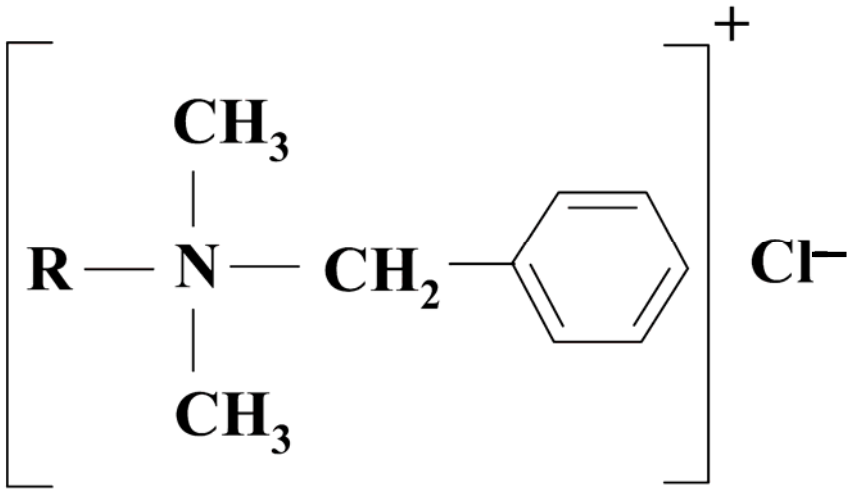

Figure 2.5.1. Chemical structure of benzalkonium chloride (BC). R represents a monoalkyl chain, length varying in carbon number (50% C_{14}, 40% C_{12}, and 10% C_{16}) [adapted with permission from Ioannou et al. (2007)].

Chaplin (1952) reported that the lipoprotein content on cell surfaces became significantly increased in BC-resistant *S. marcescens*. Similarly, an increase in the lipid content of BC-resistant *Enterobacter cloacae* and *P. aeruginosa* was also reported (Nishikawa et al., 1979; Sakagami et al., 1989). In *P. aeruginosa*, the content of phospholipids and fatty and neutral lipids in the cell walls of a BC-resistant strain was higher than those in the cells of the BC-susceptible strain; correspondingly, the amounts

of BC adsorbed to lipid components of cell walls of the BC-resistant strain were lower than those for the BC-susceptible strain (Sakagami et al., 1989). The authors concluded that the ability of BC to permeate the cell wall of *P. aeruginosa* was reduced because of the increase in cellular fatty acids, and thus the resistance to BC was mainly the result of increases in the contents of phospholipids and fatty and neutral lipids. Richards and Cavill (1976) observed using electron microscopy that a lethal concentration (800 μg ml^{-1}) of BC stripped the outer cell membrane off of *P. aeruginosa* cells, that cells retained their shape after the outer membrane was removed, and that the peptidoglycan layer remained intact.

2.6. *Salmonella* spp.

A. A. Gärtner, in 1888, isolated from meat incriminated in a large food-poisoning outbreak a bacterium subsequently named *Salmonella enteritidis*. The genus *Salmonella* was named in 1900 after a U.S. Department of Agriculture bacteriologist, Dr. Salmon, who first described a member of the group, *Salmonella choleraesuis* (Hartman, 1997). *Salmonella* spp. are well known pathogens and human salmonellosis is an important zoonotic infection that causes widespread morbidity and economic loss (Wray and Davies, 2003; Fluit, 2005). *Salmonellae* are typical members of the family *Enterobacteriaceae*; facultatively anaerobic Gram-negative bacilli able to grow on a wide range of relatively-simple media and distinguished from other members of the family by their biochemical characteristics and antigenic structure. Their normal habitat is the animal intestine (Lucas and Lee, 2000; Chart, 2002). There are over 2,500 different antigenic types (serovars or serotypes) of genus *Salmonella*, as determined based on their somatic (O) and flagellar (H) antigens (Chart, 2002; Wray and Davies, 2003; Cai et al., 2005). Many serovars are host-specific; those causing infections in man might not cause disease in animals and *vice versa*. Certain serovars are major causes of foodborne infection worldwide. Most infections are relatively benign and restricted to the intestinal tract, causing gastroenteritis and short-lived diarrhea, but some *Salmonellae* cause life-threatening systemic disease (e.g., typhoid fever) (Winfield and Groisman, 2003).

Although primarily intestinal bacteria, *Salmonella* are widespread in the environment and commonly found in farm effluents, human sewage, and in any material subject to fecal contamination. Salmonellosis has been recognized in all countries but appears to be most prevalent in areas with intensive animal husbandry, especially poultry and swine production. The disease can affect all species of domestic animals; however, young animals and pregnant animals are most susceptible. Many animals might also be infected without showing signs of illness (Wray and Davies, 2003). There are reports of various *Salmonellae* being extensively isolated from wild-living avian species such as passerines, gulls, owls, and waterfowl (Refsum et al., 2002). In the UK, annual isolations of selected serotypes from man almost tripled between 1981 and 1988. This dramatic increase was due largely to the emergence of strains belonging to *Salmonella* serovar Enteritidis, which peaked in 1997–98 and continues to be the most isolated serovar. In developing countries in which large-scale farming and processing of food animals has not been established, *Salmonellae* are not as important a cause of community-acquired diarrhea. However, infections with *Salmonella* serovar Typhi and Paratyphi, which are mainly encountered as imported infections in developed countries, remain prevalent in other parts of the world (Chart, 2002).

Salmonella has enhanced adaptability and survival in the external environment (soil, water, and on a variety of surfaces) relative to *E. coli*, which promotes its transmission and infection to a new host (Winfield and Groisman, 2003). The ability of *Salmonella* to respond effectively to the environmental changes by mounting a stress response is important in their survival in the food chain just like any other foodborne pathogen (Humphrey, 2004).

2.6.1. Classification and nomenclature of *Salmonella* spp.

Salmonella has been divided into two species based on molecular relationships, and they are *Salmonella enterica* and *Salmonella bongori* (Reeves et al., 1989; Rotger and Casadesús, 1999; Lucas and Lee, 2000). The genus *Salmonella* has a large number of named serovars, but most belong to *Salmonella enterica*. *S. enterica* can be divided into a number of subspecies and these can be divided into serovars, which might display different phage types. *S. enterica* subspecies are: *enterica* (I), *salamae* (II), *arizonae*

(IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Fluit, 2005). *Salmonella bongori* is listed as subspecies V, even though this is a separate species (Fierer and Guiney, 2001; Fluit, 2005). The complete correct designation is, for example: *Salmonella enterica* subspecies *enterica* serovar Enteritidis, but this is usually abbreviated to *Salmonella* serovar Enteritidis (*S.* serovar Enteritidis) or simply *S.* Enteritidis (Chart, 2002; Fluit, 2005).

Subspecies I (*enterica*) includes nearly 1,400 serovars, some of which are commonly isolated from infected birds and mammals, including humans, and are responsible for most *Salmonella* infections in humans; the other subspecies mainly colonize cold-blooded vertebrates (D'Aoust, 1997; Rotger and Casadesús, 1999; Lucas and Lee, 2000). The serovars might be composed of several phage types. Isolates, which are pathogenic to man belong to subspecies I, but not all serovars, subspecies, or species are pathogenic. A variety of virulence factors have been described for *Salmonella*, some of which appear to have a broad distribution, whereas others appear to be present in a limited number of serovars or even strains (Fluit, 2005). Certain serotypes are a major cause of foodborne infection worldwide. Most infections are relatively benign and restricted to the intestinal tract, causing a short-lived diarrhea, but some *Salmonellae* cause life-threatening systemic diseases, such as typhoid fever and paratyphoid fever (Chart, 2002).

2.6.2. *Salmonella* spp. as foodborne pathogens

Salmonelloses remain a major problem worldwide (Tietjen and Fung, 1995; Haeghebaert et al., 2003). For example, one of the worst food poisoning incidents in the history of the United States occurred in 1985 when 16,284 cases and 7 deaths were documented when pasteurized milk somehow became contaminated with *Salmonella* serovar Typhimurium. In 1994, this was exceeded by a national outbreak of *Salmonella* serovar Enteritidis affecting 225,000 people who consumed ice cream products (Hennessy et al., 1996; Hartman, 1997). *S. enterica* ranks as the leading cause of foodborne outbreaks worldwide (Tietjen and Fung, 1995; Humphrey, 2004; Maurer and Lee, 2005). The knowledge that very low numbers of *Salmonella* cells can be infectious prompted both traditional (selective enrichment, biochemical tests, and serological

confirmation) as well as advanced (miniaturized biochemical assay kits, new media formulations, automated instrumentation, DNA/RNA probes, antibody-dependant assays, and polymerase chain reaction) diagnostic methods for *Salmonella* being routinely used in the food industries (Tietjen and Fung, 1995).

Salmonella can be isolated from poultry processing equipment, especially in the slaughter and evisceration area (Helke et al., 1993; Joseph et al., 2001; Chmielewski and Frank, 2003). The poultry processing operation is a wet environment and therefore ideal for biofilm formation; little information is available on the presence of *Salmonella* in biofilms in food processing environments (Chmielewski and Frank, 2003). However, various studies (Helke and Wong, 1994; Jones and Bradshaw, 1997; Joseph et al., 2001) showed that *Salmonella* can attach and form biofilms on surfaces found in food processing plants, including plastic, cement, and stainless steel (Chmielewski and Frank, 2003).

2.6.2.1. Significance and economics of salmonellosis

It has been reported that more than 1.3 billion cases of human salmonellosis occur worldwide annually, resulting in three million deaths (Pang et al., 1995; Humphrey, 2004). There are roughly 1.4 million cases of salmonellosis in the United States each year, with approximately 600 deaths (Humphrey, 2004; Swanson et al., 2007). The direct and indirect economic impact of these illnesses run into the billions of dollars. In USA, *Salmonella* serovar Enteritidis has resulted in losses of more than 500 million dollars annually as a consequence of infections in humans and associated medical costs, as well as lost productivity to the egg and meat industries (Frenzen et al., 1999; Fadl et al., 2002; Food and Drug Administration, 2004).

2.6.3. *Salmonella enterica* serovar Enteritidis

Human salmonellosis caused by *Salmonella* serovar Enteritidis increased worldwide beginning in the mid-1970s and by 1990, this serovar displaced *Salmonella* serovar Typhimurium as the primary cause of the illness in the world (Baumler et al., 2000; Guard-Petter, 2001). *Salmonella* serovar Enteritidis causes gastroenteritis associated with a high mortality rate in the absence of appropriate antibiotic treatment

(Latasa et al., 2005). In France, *Salmonella* serovar Enteritidis represented 39% of human salmonellosis cases in 2001 (Haeghebaert et al., 2003). This is mainly because of the unique ability of *Salmonella* serovar Enteritidis to contaminate eggs without causing any discernible illness in the infected birds. *Salmonella* serovar Enteritidis is currently the only *Salmonella* serovar that causes frequent human illness associated with egg contamination, which determines its unique threat to food safety (Guard-Petter, 2001). During 1997, *Salmonella* serovar Enteritidis accounted for 85% of all cases of human salmonellosis in Europe; incidence has since declined from this peak. The phage type (PT) 4 *Salmonella* serovar Enteritidis predominated during the pandemic, but other phage types were also detected (Guard-Petter, 2001). The infection route to humans involves colonization, survival, and multiplication of the pathogen in the hen-house environment, the bird, and finally, the egg. The altered growth patterns and specific cell surface characteristics contribute to the adaptation of *Salmonella* serovar Enteritidis to these diverse environments (Guard-Petter, 2001).

Salmonella serovar Enteritidis resembles *Salmonella* serovar Typhimurium (primary host of *Salmonella* serovar Typhimurium is rodents) with respect to known virulence mechanisms central to mammalian cell invasion, survival, and multiplication in the host. Both pathogens share the highly conserved pathogenicity island-encoded type III secretion systems and virulence effector proteins, both harbor a large virulence plasmid, both are motile, and have a galactose-rhamnose-mannose repeating subunit of the lipopolysaccharide (LPS) O-chain backbone connected with dideoxyhexose that determines serovar specificity (Galan and Curtiss, 1991; Reeves, 1993; Jones and Falkow, 1996; Ochman and Groisman, 1996; Blanc-Potard et al., 1999; Marcus et al., 2000; Guard-Petter, 2001). However, it is unclear as to how *Salmonella* serovar Enteritidis specially follows the human infection route, while it is also possible for this pathogen to successfully contaminate and grow in egg contents (Guard-Petter, 2001). *Salmonella* serovar Enteritidis has been shown to generate a remarkable degree of strain heterogeneity, suggesting that a complex network of characteristics might underlie its diverse behavior (Guard-Petter, 2001).

The LPS obtained from *Salmonella* serovar Enteritidis differs significantly between strains in the degree of glucosylation that occurs in the O-chain region, ranging

from a lack of glucosylation in stored isolates to an exceedingly high degree of glucosylation in fresh isolates from animal tissues (Parker et al., 2001a). The glucosylated LPS O-chain of *Salmonella* serovar Enteritidis appears to contribute to the EPS of biofilms that is also rich in flagella and fimbria (Guard-Petter et al., 1996; Humphrey et al., 1996; Allen-Vercoe et al., 1997; Guard-Petter, 2001). Single nucleotide polymorphisms (SNPs) have been linked to LPS O-chain microheterogeneity and to the source of the isolate, and strain heterogeneity (Guard-Petter, 2001; Parker et al., 2001a). *Salmonella* serovar Enteritidis also produces various other types of mutants that are recoverable from the environment; undirected genetic mutation in *Salmonella* might be a response to environmental stressors. It is reasonable that some of these mutations might enhance survival and fitness of the pathogen, whereas others are without any consequences or may even be deleterious (Kolter et al., 1993; Allen-Vercoe et al., 1998; Humphrey et al., 1998; Guard-Petter et al., 1999; Massey et al., 1999).

Mutation of *flhD* (part of the *flhDC* master operon regulator of flagellar biosynthesis) to eliminate flagellation in *Salmonella* serovar Enteritidis unexpectedly enhanced oral invasiveness in poultry, whereas mutation of *fliC* (the flagellin structural gene) did not (Guard-Petter, 2001). Alteration in gene expression from the flagellar master operon affects other cell functions, such as cell division and possibly virulence (Pruss et al., 1997; Claret and Hughes, 2000a, b). Thus, the ability of some *Salmonella* serovar Enteritidis strains to significantly alter cell division is considered the bacterial characteristic that is most likely to contribute to enhanced oral invasiveness, and the ability of this pathogen to alter regulation between extremes of flagellation enhances its pathogenicity overall (Guard-Petter, 2001).

Salmonella serovar Enteritidis has been shown to be capable of *luxR*-regulated high cell density growth; the variants of *Salmonella* serovar Enteritidis that grow at high cell density are more invasive and pathogenic, especially because of the cell density-determined expression of virulence genes in the population (Guard-Petter, 1998, 2001). The cells undergo a striking morphological change (i.e., cellular elongation) during high cell density growth. Finding that *Salmonella* serovar Enteritidis undergoes acyl-homoserine lactone (AHL)-regulated high cell density growth suggests that

epidemiological surveys might be indirectly detecting the emergence of quorum-sensing, virulence-enhanced *Salmonella* serovars (Guard-Petter, 2001).

2.6.3.1. *Salmonella* serovar Enteritidis biofilm formation

It has been reported in Gram-negative bacteria that, in general, mutations in genes involved in flagellar-mediated motility, twitching motility, synthesis of EPS, quorum-sensing, outer membrane adhesins, as well as global regulators of gene expression, cause impaired biofilm formation (O'Toole et al., 2000; Solano et al., 2002). Throughout its lifecycle, *Salmonella* serovar Enteritidis has to survive inside the host and in the external environment, withstanding different regimens of nutrient availability, osmotic stress, pH, and temperature (Latasa et al., 2005). It is recognized that part of the ecological success of bacteria that have to face such highly-variable environmental conditions lies in their ability to grow as surface-attached biofilms, embedded in a protective extracellular matrix (Costerton et al., 1995; Davey and O'Toole, 2000; Latasa et al., 2005).

Salmonella serovar Enteritidis has been shown to form biofilms on materials of different nature and under different growth conditions (Korber et al., 1997; Solano et al., 1998, 2002; Mangalappalli-Illathu and Korber, 2006). Zogaj et al. (2001) demonstrated that natural *Salmonella* serovar Enteritidis isolates showed the multicellular and aggregative (rdar [red, dry, and rough] morphotype) behavior previously described only in *Salmonella* serovar Typhimurium. Knocking-out the gene encoding for thin aggregative fimbriae, AgfA, resulted in inability to form a pellicle in standing culture of *Salmonella* serovar Typhimurium, indicating that thin aggregative fimbriae are one of the components of the EPS, even though the cells in the colony were still connected in an elastic fashion (Römling and Rohde, 1999). Multicellular behavior has been shown to be positively-regulated at the onset of stationary phase by *agfD*, a gene encoding a putative response regulator of thin aggregative fimbriae expression (Römling et al., 2000). Such mutants lacked all forms of multicellular behavior. RpoS was also found to regulate the production of the unknown extracellular substance (Römling et al., 2000).

Previous studies on *Salmonella* serovar Enteritidis biofilm formation have shown that, in rich medium (broth) and at room temperature (28ºC), these bacteria produce

pellicle whose matrix is mainly composed of curli or thin aggregative fimbriae and cellulose (Zogaj et al., 2001; Solano et al., 2002). Disruption of any of the two operons responsible for cellulose biosynthesis, *bcsABZC* and *bscEFG*, impaired pellicle formation and significantly increased the susceptibility of *Salmonella* serovar Enteritidis to disinfectants (Solano et al., 2002). Both curli and cellulose synthesis are coregulated by a complex regulatory network, in which the LuxR type regulator CsgD plays a key role (Römling et al., 2000). It was believed, until recently, that unlike other Gram-negative bacteria, where various surfaces or intercellular adhesion factors were shown to participate in biofilm formation, only curli and cellulose production has been described to be involved in the *S. enterica* biofilm formation process (Schembri et al., 2004; Latasa et al., 2005). Recently, it has been reported in *Salmonella* serovar Enteritidis that a large cell wall-associated secreted protein, BapA, having sequence homology with Bap (biofilm-associated protein) of *S. aureus*, has been found to be required for the biofilm formation and host colonization (Cucarella et al., 2001; Latasa et al., 2005).

Biofilm-forming *Salmonella* serovar Enteritidis isolates are considered to be more virulent. The ability of *Salmonella* serovar Enteritidis to form biofilms correlates with enhanced oral invasiveness, but not epithelial cell disruption and egg contamination (Guard-Petter et al., 1996; Solano et al., 2001). However, Parker et al. (2001b) reported that biofilm-producing *Salmonella* serovar Enteritidis might act as a 'helper' phenotype that aids access of less orally-invasive strains to the post-mucosal environment of the bird, with subsequent enhanced recovery of contaminated eggs.

2.6.4. Stress response in *Salmonella* spp.

Living cells have evolved remarkable mechanisms for maintaining homeostasis under adverse growth conditions (Bianchi and Baneyx, 1999). In *E. coli*, temperature upshifts and other types of stress induce the synthesis of heat-shock proteins belonging to the σ^{32} regulon if misfolded proteins accumulate in the cytoplasm, and to the σ^{E} regulon if damage is sustained by the outer membrane or in the periplasm (Gross, 1996; Bianchi and Baneyx, 1999). In contrast, exposure to low temperatures leads to a transient shutdown of general protein synthesis and high-level accumulation of cold-shock proteins whose Eσ^{70}-synthesized transcripts contain characteristic 5'-untranslated

regions that play a central role in post-transcriptional regulation (Thieringer et al., 1998; Bianchi and Baneyx, 1999).

S. enterica spends a good part of their life as residents of animal hosts, which are believed to be the primary habitat of this organism. *Salmonella* has genes that mediate invasion of, and survival within, host cells, including genes that promote resistance to different microbicidal host products (Winfield and Groisman, 2003). Once excreted from an animal host, *Salmonella* find themselves battling for survival, facing limited nutrient availability, osmotic stress, large variations in temperature and pH, and predation (Winfield and Groisman, 2003). It has been proposed that bacteria survive such stressful conditions by entering a VBNC state. The VBNC hypothesis describes an apparent dormant state in which bacterial cells are metabolically active but cannot be cultured by known laboratory methods; nutrient addition and temperature upshift are reportedly effective methods for reviving bacterial populations from the VBNC state (Ravel et al., 1995; Russell, 2003a; Winfield and Groisman, 2003). As an additional stress, the cells might be exposed to a wide range of antibiotics and biocides that could act as a selective pressure for the development and isolation of resistant cultures by several mechanisms (Levy, 2002; Russell, 2003a). When exposed to a harmful stress, bacteria will do all in their power to survive. The effect of a hostile agent on bacteria can be seen as producing a stress response, causing inhibition or inactivation of the cell, or resulting in tolerance/resistance of the cell (Russell, 2003a).

In order to grow, most bacteria require a moderate temperature, pH range, and high water availability (Philips et al., 1998). However, *Salmonellae* routinely survive within extreme environments by inducing specific stimulons which might promote survival (Kolter et al., 1993; Foster and Spector, 1995). Philips et al. (1998) reported that chilling invoked cell elongation and heterogeneity in heat- and acid-tolerant *Salmonella* serovar Enteritidis strain, and that this was possibly linked to its pathogenicity. *Salmonella* serovar Typhimurium has evolved a complex series of stress management response systems in order to survive various environmental stresses such as heat, starvation, low pH, oxidative stress, osmolarity, and presence of cationic peptides and iron, in which an array of regulatory proteins (Crp, RelA, RpoS, AtbR, MviA, Fur, OxyR, PhoPQ, and OmpR) provides overlapping control that links many of these

systems (Foster and Spector, 1995). It has been reported that dehydrated cells of *Salmonella* have an increased resistance against several environmental stress conditions, such as heat (Kirby and Davies, 1990). *Salmonella* are relatively resistant to dry conditions and may grow at $a_w > 0.94$ (Jay, 2000). The ability to survive air-drying on surfaces has been reported to vary between isolates of *Salmonella* (Humphrey et al., 1995; Jørgensen et al., 2000).

Van Bogelen and Neidhart (1990) reported that when antibiotics targeting the prokaryotic ribosomes were added to the growth medium of *E. coli* cultivated at 37ºC, induction of either heat-shock proteins or cold-shock proteins was observed depending on whether the A site of the ribosome was empty or occupied. Antibiotics that induced a heat-shock response (e.g., streptomycin and neomycin) were designated H-group antibiotics, while antibiotics that induced a cold-shock response (e.g., chloramphenicol and tetracycline) were designated C-group antibiotics (Bianchi and Baneyx, 1999). Bianchi and Baneyx (1999) also reported that stress responses could be used as a tool to detect and characterize the mode of action of antibacterial agents on *E. coli*. The authors reported that single-copy fusions between the *lacZ* reporter gene and *E. coli* strains containing promoters induced by cold-shock, cytoplasmic stress, or protein misfolding in the cell envelope were used to determine their ability to detect antibacterial agents while simultaneously providing information on their cellular targets.

2.6.5. Antimicrobial resistance of *Salmonella* spp.

Antibiotic resistance determinants in *Salmonella* usually are encoded on plasmids, but can also be present on the multidrug resistance region of *Salmonella* Genomic Island 1 (SGI 1) (Fluit, 2005). Antibiotic resistance among *Salmonella* is increasing; especially, the development of resistance against newer antibiotics. The increased occurrence of integrons, which can harbor a variable set of antibiotic resistance encoding gene cassettes that can be exchanged between integrons, is a major concern. Furthermore, *β*-lactamases with activity against broad-spectrum cephalosporins have been found associated with *Salmonella* integrons. There are reports that virulence and resistance plasmids of *Salmonella* form co-integrates, which could be selected by antibiotic pressure and thereby for virulence factors (Fluit, 2005).

The resistance to disinfectants might be important in the survival and persistence of *Salmonella* in feed mills and feed factories that are regularly disinfected, even though only a few reports exist on resistance of *Salmonella* to disinfectants (Møretrø et al., 2003). The authors suggested that concentrations of disinfectants and proper cleaning might be critical to inhibit persistence. Resistance among *Salmonella* from poultry abattoir to hypochlorous acid (Mokgatla et al., 1998) and increased tolerance of *Salmonella* against H_2O_2 has been reported (Seymor et al., 1996). There are reports of adaptation of *S. enterica* to BC and cross-resistance of adapted strains to various antibiotics (chloramphenicol) and biocides (chlorhexidine) (Braoudaki and Hilton, 2004, 2005). The authors concluded that cell surface hydrophobicity and the presence of active efflux systems could be attributed to the resistance of *S. enterica* to erythromycin, triclosan, and BC (Braoudaki and Hilton, 2005).

2.7. Context of the study

The influence of intrinsic factors such as genetic composition on *Salmonella* serovar Enteritidis biofilm formation has been evaluated to some extent; whereas, information on effect of various environmental (extrinsic) factors such as flow velocity, hydrodynamic disturbances, nutrient status, chemical stress etc. on biofilm formation and development is lacking. However, this topic requires much attention because of the emerging nature of this significant foodborne enteric pathogen. Thus, an investigation on various extrinsic factors influencing *Salmonella* serovar Enteritidis biofilm formation and development has been undertaken. This study is designed from the perspective of survival of *Salmonella* serovar Enteritidis as biofilms on food processing surfaces, in order to elucidate the adaptive responses/mechanisms of biofilms to conditions of nutrient laminar flow and to prolonged sub-lethal exposure to the antimicrobial agent, BC. The architectural adaptation of the biofilms has been investigated to explore the survival mechanisms in a laminar flow environment. The morphological and physiological responses of the cells in tightly-adherent (non-shearable) and loosely-adherent (shearable) biofilm regions have been studied in order to elucidate the attributes of cells that might contribute to their differential survival and adaptation. The biofilms were treated with sub-lethal concentration of BC, both in continuous and intermittent

regimens in order to evaluate the best mode of BC treatment for development of biofilm adaptive resistance to this antimicrobial agent, and thus to help elucidate the cellular processes involved in biofilm adaptive resistance. The planktonic and biofilm phenotypes respond to antimicrobial stresses in distinct manner. Thus, differential adaptive response and survival of planktonic and biofilm cells following prolonged sub-lethal exposure to BC, mediated through cellular morphological and physiological alterations, were also investigated.

3. ARCHITECTURAL ADAPTATION AND PROTEIN EXPRESSION PATTERNS OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS BIOFILMS UNDER LAMINAR FLOW CONDITIONS

3.1. Abstract

Salmonella enterica serovar Enteritidis is a significant biofilm-forming pathogen. The influence of a 10-fold difference in nutrient laminar flow velocity on the dynamics of *Salmonella* serovar Enteritidis biofilm formation and protein expression profiles was compared in order to ascertain how flow velocity influenced biofilm structure and function. Low-flow (0.007 cm sec^{-1}) biofilms consisted of diffusely-arranged microcolonies that grew until merging by ~72 h. High-flow (0.07 cm sec^{-1}) biofilms were significantly thicker (36 ± 3 μm vs. 16 ± 2 μm for low-flow biofilms at 120 h) and consisted of large bacterial mounds interspersed by water channels. Lectin-binding analysis of biofilm exopolymers revealed a significantly higher ($P < 0.05$) proportion of *N*-acetyl galactosamine (GalNAc) in low-flow biofilms (55.2%), relative to only 1.2% in high-flow biofilms. Alternatively, the proportions of α-L-fucose and *N*-acetyl glucosamine (GlcNAc2) – *N*-acetyl neuraminic acid (NeuNAc) polymer conjugates were significantly higher ($P < 0.05$) in high-flow biofilms (69.1% and 29.6%, respectively) than low-flow biofilms (33.1% and 11.7%, respectively). Despite an apparent flow

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This chapter is reproduced from a manuscript submitted for publication. Copyright has not been transferred. The manuscript is co-authored by J. R. Lawrence, G. D. W. Swerhone, and D. R. Korber. All experimental works with the exception of confocal laser microscopy (lectin-binding experiment) data analyses and mass spectrometry analyses were carried out by me. In addition, I wrote the initial draft of the manuscript. Minor modifications were made to the submitted version to maintain thesis format and style.

rate-based physiologic effect on biofilm structure and exopolymer composition, no major shift in protein expression was observed between the low-flow and high-flow biofilms, and notably did not include any response involving the stress response proteins, DnaK, SodB, and Tpx. Proteins involved in degradation and energy metabolism (PduA, GapA, GpmA, Pgk, and RpiA), RNA and protein biosynthesis (Tsf, TufA, and RpoZ), cell processes (Crr, MalE, and PtsH), and adaptation (GrcA), and some hypothetical proteins (YcbL and YnaF) were up-regulated in both biofilm systems. The results of this study indicate that *Salmonella* serovar Enteritidis biofilms altered their architecture in response to flow, thereby assuming a structure that minimized overall biofilm stress. The ability to minimize stress within biofilms likely represents an evolutionary response to life on surfaces and helps explain how *Salmonella* serovar Enteritidis cells are able to persist under apparently hostile conditions imposed within food processing environments.

3.2. Introduction

Salmonella enterica serovar Enteritidis has emerged as one of the most important of all the foodborne pathogens, causing major outbreaks, especially in developed countries over the past 30 years (Patrick et al., 2004; Guerin et al., 2006). *Salmonella* serovar Enteritidis has resulted in losses of more than 500 million dollars annually as a consequence of infections in humans and associated medical costs, as well as lost productivity to the egg and meat industries (Frenzen et al., 1999; Fadl et al., 2002; Food and Drug Administration, 2004).

Biofilm formation by *Salmonella* serovar Enteritidis has been well-documented (Korber et al., 1997; Stepanović et al., 2003; Mangalappalli-Illathu and Korber, 2006) and it has been reported that nearly all (97%) of 204 *Salmonella* serovar Enteritidis isolates obtained from various sources may form biofilms (Solano et al., 2002). The attachment to, and subsequent development of *Salmonella* biofilms on food processing surfaces may have significant economic consequences, since persistent biofilms serve as a recurring source of pathogens in food products (Stepanović et al., 2003). In systems dominated by laminar flow, molecular diffusion is the primary mechanism whereby nutrients and wastes are transported into and out of the biofilm matrix (Bryers, 1987;

Davey and O'Toole, 2000). While there have been recent attempts to generalize the effects of fluid flow velocity on biofilm developmental processes, it should be noted that considerable variability in biofilm formation has been reported for different bacteria grown under various hydrodynamic regimens. It has further been hypothesized that cellular automaton models are applicable for describing the development and architecture of microbial biofilms; the interaction between substrate concentration, substrate gradient, and detachment forces might significantly influence the biofilm structure resulting in a system with overall reduced stress (Van Loosdrecht et al., 1997; Wimpenny and Colasanti, 1997).

Although molecular mechanisms mediating attachment and biofilm formation in *Salmonella* spp. are not completely understood, it is recognized that various genes and their products are either up-regulated or down-regulated in response to biofilm formation. For example, the *agf*D promoter encoding a putative response regulator of thin aggregative fimbriae expression is involved in biofilm formation by *Salmonella* serovar Typhimurium (Römling et al., 2000; Gerstel and Römling, 2001; Stepanović et al., 2003). Information on the regulatory mechanisms controlling the expression of *agf*D, and therefore biofilm formation, is limited even though multicellular behavior has been shown to be positively regulated at the onset of the stationary phase by *agf*D (Römling et al., 1998, 2000; Gerstel and Römling, 2001). Two genetic operons, namely *bcs*ABZC and *bcs*EFG, are also required for the biosynthesis of cellulose which is a constituent of *Salmonella* serovar Enteritidis EPS, suggesting that cellulose production is factor in the survival and proliferation of *Salmonella* serovar Enteritidis biofilms associated with surface environments (Solano et al., 2002). It has also been reported in *E. coli* that 22% of genes were up-regulated in the biofilm state, and 16% were down-regulated (Prigent-Combaret et al., 1999). Evidence exists that up-regulation and down-regulation of a number of genes occur in surface colonizing cells following initial interaction with the substratum (Donlan, 2002). Notably, despite hypotheses that the biofilm contains zones where conditions would be presumed to be stressful (e.g., cells in the stationary phase), no work has demonstrated large-scale expression of stress genes.

The influence of hydrodynamic conditions on the formation and maintenance of biofilms formed by *Salmonella* spp. is not well understood (Stepanović et al., 2003), and could be significant for the development of resistant phenotypes responsible for resistance to antimicrobial agents on food processing surfaces. This study used nutrient flow velocity as a tool to study the influence of low laminar flow, in order to simulate the effect of nutrient flow conditions that occur in routine food processing environments, on: (i) the development of biofilm architecture, (ii) the biofilm EPS composition, and (iii) the protein expression profiles of *Salmonella* serovar Enteritidis biofilms.

3.3. Materials and methods

3.3.1. Media and chemicals

Tryptic Soy Agar (TSA), Standard Plate Count Agar (SPCA), and Trypticase Soy Broth (TSB) were purchased from BBL (Becton Dickinson, Cockeysville, MD); magnesium chloride ($MgCl_2$), phenylmethylsulphonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), DNase, RNase A, fluorescein sodium salt, bromophenol blue, DL-dithiothreitol (DTT), and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO); sodium chloride was from EM Science (Gibbstown, NJ); EDTA was from J. T. Baker Chemical Co. (Philipsburg, NJ); glycerol, sodium dodecyl sulphate (SDS), Tris base, and urea were purchased from Life Technologies (Grand Island, NY); *Bac*Light™ Live/Dead Viability Probe was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada); and immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0), Immobiline DryStrip gels, and PlusOne™ Protein Silver Staining Kit were purchased from GE Healthcare Bio-Sciences Inc. (Baie d'Urfé, QC, Canada). The plant lectins *Ulex europaeus* and *Triticum vulgaris* conjugated with fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) respectively were purchased from Sigma Chemical Co., as was the lectin *Glycine max*. The cyanine dye (CY5) labeling for *Glycine max* was performed using a commercial labeling kit according to the manufacturer's instructions (Research Organics, Cleveland, OH).

3.3.2. Bacteria and culture conditions

Salmonella enterica serovar Enteritidis ATCC 4931 (hereafter referred to as *Salmonella* serovar Enteritidis) was cultured from a frozen stock on TSA plates overnight at 37ºC. Cells in the mid-log phase of growth were obtained by transferring a loopful of colony material from TSA plates to 50 ml of 10% [wt/vol] TSB in an Erlenmeyer flask and incubating on a gyratory shaker (150 ± 5 rpm) held at room temperature (RT; 21 ± 2ºC) as batch culture for approximately 12 h. These cells, which were previously determined to be in the mid-log phase of growth via growth curve experiment, were used to inoculate flow cells. Planktonic cells for control experiments were grown in continuous culture in an Erlenmeyer flask held on a gyratory shaker (150 ± 5 rpm) and incubated at RT for 168 h; the total culture volume was set at 175 ml. Nutrient medium (10% [wt/vol] TSB) was added and removed continuously at the rate of 25 ml h^{-1} resulting in a dilution rate of 0.14 h^{-1}. The medium was pumped into and out of the Erlenmeyer flask via silicone tubing using two Watson-Marlow peristaltic pumps (Model 202U; Watson-Marlow, Cornwall, UK), with each one set to control the input of the medium to the flask and removal of the effluent to a waste reservoir.

3.3.3. Flow cells, inoculation, and flow velocity

Multi-channel flow cells were constructed using 5 mm thick sheets of polycarbonate plastic into which were milled channels that were covered with glass coverslips, as described previously (Korber et al., 1994). Flow cells constructed out of glass slides and coverslips were used for dark-field microscopy. Flow cell channels were sterilized by flushing with 5.25% [wt/vol] sodium hypochlorite solution for 10 min. Reservoirs of sterile nutrient medium (10% [wt/vol] TSB) were connected via silicone tubing to the flow cell channels and subsequently connected to the effluent reservoir. The medium was pumped through flow cells using a Watson-Marlow peristaltic pump (*see* Figures 2.3.1 and 2.3.2). Each flow cell channel was separately inoculated with 0.5 ml mid-log phase *Salmonella* serovar Enteritidis cells, prepared as outlined above, concentrated or diluted to an optical density equivalent to 0.5 McFarland standard (1.5 X 10^8 cfu ml^{-1}). The bulk flow rates of 2.5 ml h^{-1} and 25 ml h^{-1} resulted in laminar flow velocities of 0.007 cm sec^{-1} and 0.07 cm sec^{-1}, respectively. Biofilms grown at nutrient

laminar flow velocities of 0.007 cm sec^{-1} and 0.07 cm sec^{-1} will hereafter be known as low-flow and high-flow biofilms, respectively. Corresponding Reynolds numbers (*Re*) for low-flow and high-flow conditions were determined to be 0.237 and 2.37, respectively (Vogel, 1983).

3.3.4. Steady-state growth

Steady-state growth of biofilms grown on glass and silicone surfaces was determined by assessing the pattern of dispersion of cells and the thicknesses of the biofilms (*see* below). Analysis of cell shedding or dispersion helped to quantify the extent of cell release from the glass surface and silicone tubing surfaces. The effluent from biofilms grown on 10% [wt/vol] TSB as nutrient medium at low-laminar flow and high-laminar flow velocities in either the channels of glass flow cells or on the inner surface of silicone tubing was collected aseptically, and enumerated at 12 h intervals (n = 4). The effluent was serially diluted and plated on SPCA over a 168 h period in order to define the point at which the biofilms grown on the two different substrates reached a "pseudo-steady state" condition (Fux et al., 2004). Colonies were enumerated after overnight growth at 37ºC and the pattern of cell release vs. time was used to determine when the biofilms reached the "pseudo-steady state" condition.

3.3.5. Dark-field microscopy

A Zeiss model III RS microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with a 10X dark-field (0.22 numerical aperture) objective lens and a cooled CCD camera (Sensys model 1401E; Photometrics, Tucson, AZ) controlled by PC-based RS Image version 1.7.3 software (Roper Scientific Inc., Trenton, NJ) was used to collect images for low-power montage reconstruction. Montages of approximately 1 X 10^6 μm^2 in total area were assembled using Adobe Photoshop version 7.0 software (Adobe Systems Inc., Mountain View, CA).

3.3.6. Biofilm thickness measurements

The thickness of biofilms was measured in μm using a computer controlled, motorized *z*-axis stepper motor and manual focusing using a Nikon Microphot-FXA

microscope (Nikon Corp., Tokyo, Japan) (Korber et al., 1994). Fifteen random fields were assessed for each biofilm with five separate thickness values obtained per field (n = 75). These values were averaged to obtain the thickness of each biofilm. The thickness measurements at each time interval (24 h) are the average of 225 thickness measurements made at random locations from three biofilms replicated experimentally.

3.3.7. Confocal laser scanning microscopy (CLSM), fluorescent probes, and digital image analyses

Optical thin sections (OTSs) were acquired for estimating biofilm biomass and viability of cells using a Bio-Rad MRC-600 Lasersharp fluorescence scanning confocal laser system (Bio-Rad Microscience, Hemel Hempstead, UK now Carl Zeiss MicroImaging GmbH, Jena, Germany) mounted on a Nikon Microphot-FXA microscope. Optical thin sections (each a total of 8,067 μm^2) from 5 biofilm depths (0, 3.7, 7.4, 11.1, and 14.8 μm, where 0 μm represents the biofilm-substratum interface) were collected for each of 15 biofilm sampling locations. Fluorescein was used for negative staining of biofilms and subsequent CLSM biomass estimation (Caldwell et al., 1992b). To determine bacterial viability as influenced by the flow stress, biofilms were stained with the *Bac*Light™ Live/Dead Viability Probe and assayed using dual-channel CLSM, and their fluorescence response was quantified as follows (Korber et al., 1996, 2002; Webb et al., 2003). Single-channel or dual-channel images were acquired in either the *xy* or vertical *xz* plane of analysis. The analyses of images for biofilm biomass and viability estimations were performed using MacIntosh™-based NIH Image version 1.63f software (National Institutes of Health, Bethesda, MD).

The application of CLSM coupled with fluor-conjugated lectins to quantitatively probe the EPS components within biofilm communities is well documented (Neu and Lawrence, 1999; Neu et al., 2001). A preliminary survey examining eight different fluor-lectin conjugates showed that *Glycine max*-CY5, *Triticum vulgaris*-TRITC, and *Ulex europaeus*-FITC had the most extensive binding to glycoconjugate residues of *Salmonella* serovar Enteritidis biofilms (Table 3.3.1). The fluor-conjugated lectins *Glycine max*-CY5, *Triticum vulgaris*-TRITC, and *Ulex europaeus*-FITC were

Table 3.3.1. List of lectins used in the preliminary lectin-binding experiments

Fluorescent labeled lectins	Glycocojugates detected
Bandiera simplicifolia-TRITC	Terminal *α*-D-galactosyl and *N*-acetyl galactosaminyl residues
Glycine max-CY5	*N*-acetyl galactosamine
Naja mossambica mossambica-Alexa 488*	Heparin
Triticum vulgaris-TRITC	*N*-acetyl glucosamine and *N*-acetyl neuraminic acid
Ulex europaeus-FITC	*α*-L-fucose
Ulex europaeus II-CY5	*N*,*N*-diacetyl chitobiose
Vicia sativa-Alexa 568*	Glucose and Mannose
Vicia villosa-FITC	*N*-acetyl galactosamine

* Alexa dyes were from Invitrogen Canada Inc., Burlington, ON, Canada (formerly Molecular Probes, Inc.).

subsequently used in combination (triple lectin labeling) for *in situ* analyses of EPS composition, as described previously (Neu et al., 2001). *N*-acetyl galactosamine (GalNAc) residues in the EPS were detected by the binding of *Glycine max*-CY5 conjugate, *α*-L-fucose (fucose) residues were detected by the binding of *Ulex europaeus*-FITC conjugate, and *N*-acetyl glucosamine (GlcNAc2) and *N*-acetyl neuraminic acid (NeuNAc) residues were detected by the binding of *Triticum vulgaris*-TRITC conjugate. An analysis of the binding of fluorescent lectins was carried out with a Bio-Rad MRC-1024 confocal laser scanning microscope mounted on a Nikon Microphot-SA microscope. CLSM OTSs were collected in each of the three channels to determine EPS biomass at various depths. Digital image analyses were performed using NIH Image version 1.61 with macros written for semi-automated quantification, as described previously (Lawrence et al., 1998; Neu et al., 2001). Image analyses and calculations of lectin-binding volumes were carried out using the equations, as reported previously (Neu et al., 2001). Three color red-green-blue projections of the biofilms were prepared using Adobe Photoshop version 7.0.

3.3.8. Sample preparation for 2D-PAGE of total cellular proteins

Biofilms were aseptically scraped from the flow cell channels using disposable loops. Both biofilm and planktonic cells were pelleted by centrifuging at 4,000 rpm (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 5 min. The 2D-PAGE sample preparation was carried out as detailed previously (Mangalappalli-Illathu and Korber, 2006). Following protein extraction, the dye-binding assay of Bradford (1976) was then performed to quantify the protein concentration using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

3.3.9. 2D-PAGE and analyses of protein spots

Protein extracts were subjected to high-resolution 2D-PAGE, as described previously (O'Farrell, 1975; Görg et al., 2000). Isoelectric focusing was performed using Immobiline DryStrips (p*I* 4.0 to 7.0), each strip containing 10 µg of protein in conjunction with a Multiphor II electrophoresis unit (GE Healthcare Bio-Sciences Inc.). Equilibrated isoelectric focused strips were placed on a 14% [wt/vol] SDS-

polyacrylamide gel for second-dimension electrophoresis with a Mini-Protean II electrophoresis system (Bio-Rad Laboratories) at a constant 100 V at RT. After electrophoresis, the gels were silver stained in accordance with the manufacturer's instructions, scanned on an Epson 1200C scanner with a transparency adapter as 8-bit grayscale 300 dpi images, and stored. Differentially-expressed proteins were then detected and quantified from the stored images using Phoretix^{TM} 2D version 2004 analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). An increase in the protein spot volume of 1.5-fold or more was interpreted as up-regulation, whereas a decrease in the spot volume of 1.5-fold or more was interpreted as down-regulation.

3.3.10. Protein identification

LC-MS/MS analysis was performed using a capLC interfaced to a Q-ToF Ultima Global hybrid tandem mass spectrometer fitted with a *Z*-spray nanoelectrospray ion source (Waters-Micromass, Manchester, UK). Protein spots for MS analysis were collected from freshly-prepared 2D-PAGE gels that stained by a modified silver staining protocol provided by GE Healthcare Bio-Sciences Inc. Protein spots of interest were excised from the gel, destained, and in-gel digested with trypsin in accordance with the established protocols for the MassPrep robotic workstation (Waters-Micromass). LC-MS/MS data were processed using ProteinLynx software (Waters-Micromass) and searched against the NCBInr, MSDB, or Swiss-Prot/TrEMBL protein databases using Mascot Search (Matrix Science Ltd., London, UK). The biological function(s) of each protein identified were determined from the Wellcome Trust Sanger Institute and PUMA2 databases, as reported previously (Mangalappalli-Illathu and Korber, 2006).

3.3.11. Experimental replication

All experimental data represent the average of at least three experiments. Differential protein expression of low-flow and high-flow *Salmonella* serovar Enteritidis biofilms was determined from averaged spot volumes from four gels replicated experimentally, with a maximum variation of 30% in spot volume between the gels.

3.3.12. Statistical analyses

Planktonic cell dispersion data and biofilm thickness, biomass, and viability data were analyzed using SAS statistical software (version 9.1.3; SAS Institute Inc., Cary, NC), and the Fisher's Least Significant Difference (LSD) method was used to test for significant ($P < 0.05$) differences.

3.4. Results

3.4.1. Influence of flow velocity on the steady-state growth of biofilms

Effluent from glass flow cells and silicone tubing was collected aseptically at 12 h intervals, serially diluted and plated during 168 h in order to enumerate the number of released cells present in the effluent. After 12 h of growth on silicone tubing surfaces the number of cells in the effluent was approximately 1.6 X 10^4 cfu ml^{-1} and 4.8 X 10^2 cfu ml^{-1} for low-flow and high-flow velocities, respectively; whereas, the number of cells per milliliter released from glass surfaces at low-flow and high-flow were much higher (5.1 X 10^7 cfu ml^{-1} and 6.1 X 10^6 cfu ml^{-1}, respectively) (Figure 3.4.1). The number of cells released per sampling interval increased continuously after the 12 h sampling period until a steady-state release of cells was achieved at ~96 h in the case of silicone and glass surfaces with the release of ~3.5 X 10^8 cfu ml^{-1} in both low-flow and high-flow velocities. The number of released cells remained relatively constant over the 96 h to 168 h period.

3.4.2. Influence of flow velocity on the architecture of biofilms

Biofilms cultivated under low-flow and high-flow conditions initially consisted of discrete microcolonies that developed into diffuse growth via the coalescence of microcolonies under low-flow conditions by 192 h; under high-flow conditions over the same period microcolonies developed into a considerably thicker growth with large bacterial mounds interspersed by water channels (Figure 3.4.2). The average thickness (n = 225) of *Salmonella* serovar Enteritidis biofilms steadily increased until about 144 h under low-flow conditions, and 120 h under high-flow conditions (Figure 3.4.3). The

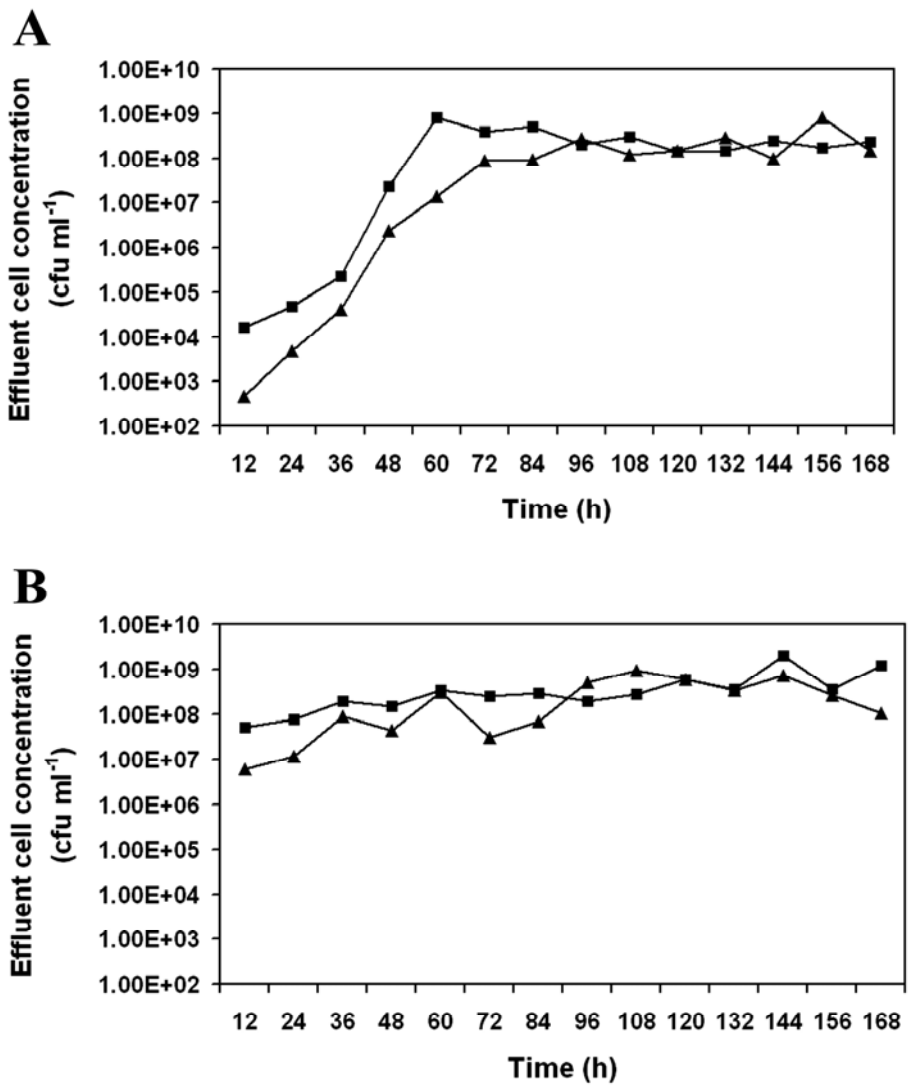

Figure 3.4.1. Pattern of release of planktonic cells from the developing low-flow and high-flow biofilms of *Salmonella* serovar Enteritidis on (A) silicone tubing surface and (B) glass surface over the 168 h growth period (n = 4). The low-flow and high-flow biofilms reached a steady-state release of cells on both the surfaces at ~96 h of growth. The symbols (—■—) and (—▲—) indicate the patterns of plankonic cell release from the low-flow and high-flow biofilms, respectively.

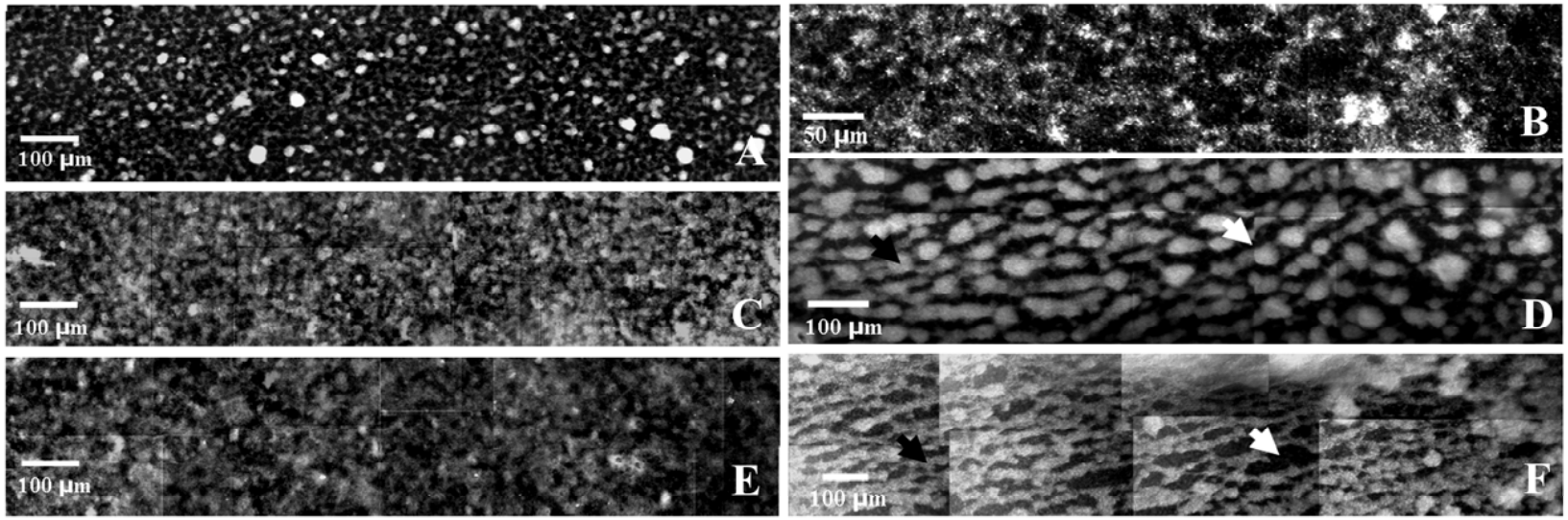

Figure 3.4.2. Low-power dark field micrograph montages showing the architectural details of (A) 48 h low-flow biofilms and (B) high-flow biofilms, (C) 144 h low-flow biofilms and (D) high-flow biofilms, and (E) 192 h low-flow biofilms and (F) high-flow biofilms. The microcolonies coalesced to form diffuse growth in low-flow conditions, whereas, biofilms developed into thick growth with large bacterial mounds (indicated by black arrows) interspersed by water channels (indicated by white arrows) in high-flow conditions.

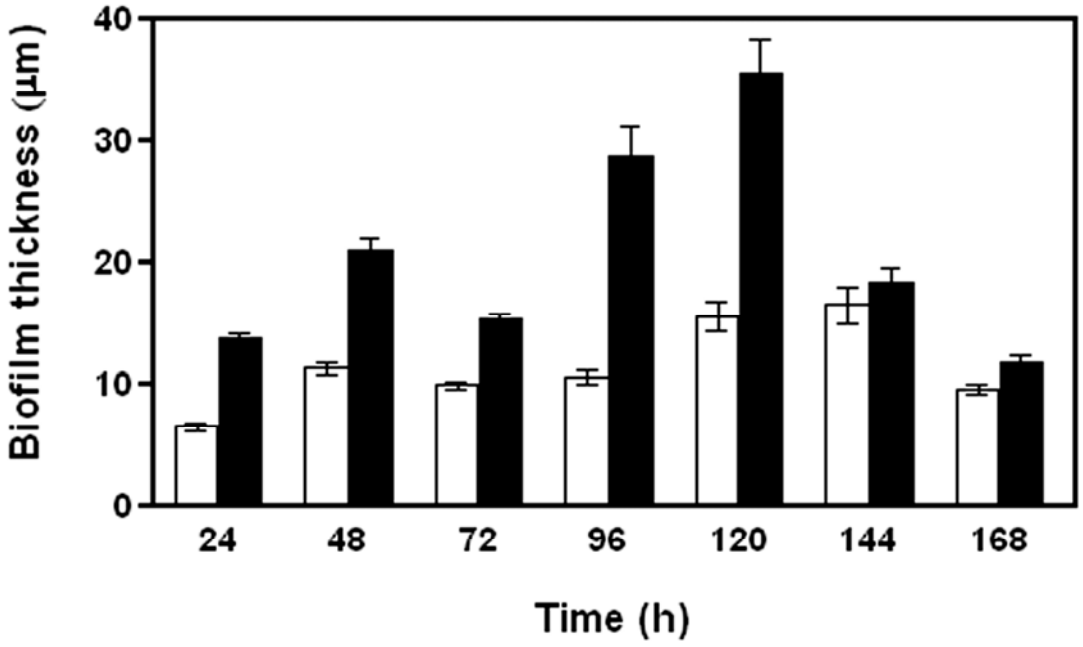

Figure 3.4.3. Average thickness measurements of low-flow and high-flow biofilms over the 168 h growth period. The thickness measurements at each time interval are the average of 225 thickness measurements made at random locations of three biofilms replicated experimentally. The symbols (▭) and (■) indicate low-flow and high-flow biofilm thickness values, respectively. The error bars indicate the standard error of the mean.

average thickness of biofilms grown at high-flow varied over time, but generally increased over the first 120 h. Bacterial mounds in high-flow biofilms were on average 36 ± 3 μm (± standard error) in thickness, ranging from minimum and maximum thickness values of 12 and 60 μm, respectively (Figure 3.4.3); the high-flow biofilms were twice as thick as *Salmonella* serovar Enteritidis biofilms grown at low-flow velocities (16 ± 2 μm). The standard deviation of thickness measurements were 16 ± 10 μm and 36 ± 24 μm for low-flow and high-flow biofilms at 120 h, respectively. The biofilms grown under low-flow conditions were less thick and less variable (Figure 3.4.3), whereas the biofilms grown at high-flow conditions were of variable thickness at different locations and found to contain water channels, mounds, and branching structures (Figure 3.4.2). The average maximum width of water channels in high-flow *Salmonella* serovar Enteritidis biofilms increased from 25.3 ± 5.3 μm (± standard deviation) at 144 h, to 33.2 ± 10.3 μm by 192 h (Figure 3.4.2D and F).

The total biofilm biomass distribution was calculated by quantifying the percentage of area covered by negatively-stained cells per microscopic field (8,067 μm^2) using NIH Image. It was determined that the biomass at the biofilm-substratum interface (0 μm OTS depth) of high-flow biofilm was generally equal to that of the low-flow biofilms (Figure 3.4.4). At the 3.7 μm optical section depth, there was often a significantly higher ($P < 0.05$) amount of biomass in the high-flow biofilms. There was significantly less biofilm biomass in the outer layers (7.4, 11.1, and 14.8 μm OTSs) than near the biofilm-substratum interface for both low-flow and high-flow biofilms; however, the greater overall thickness of high-flow biofilms reflected the presence of less dense cell material held in place by non-staining polymeric material.

The viability of the cells was assessed by staining the biofilms with the *Bac*Light™ Live/Dead Viability probe (Figure 3.4.5). The viable and non-viable biomass at the 0, 3.7, 7.4, 11.1, and 14.8 μm OTS depths were analyzed as the percent of area covered by viable and non-viable biomass. Overall, the proportion of viable biomass was 14 to 20-fold higher than the proportion of non-viable biomass both in low-flow and high-flow biofilms during the 168 h growth period. For example, after 96 h growth at the biofilm-substratum interface (0 μm OTS depth), the ratio of viable to non-

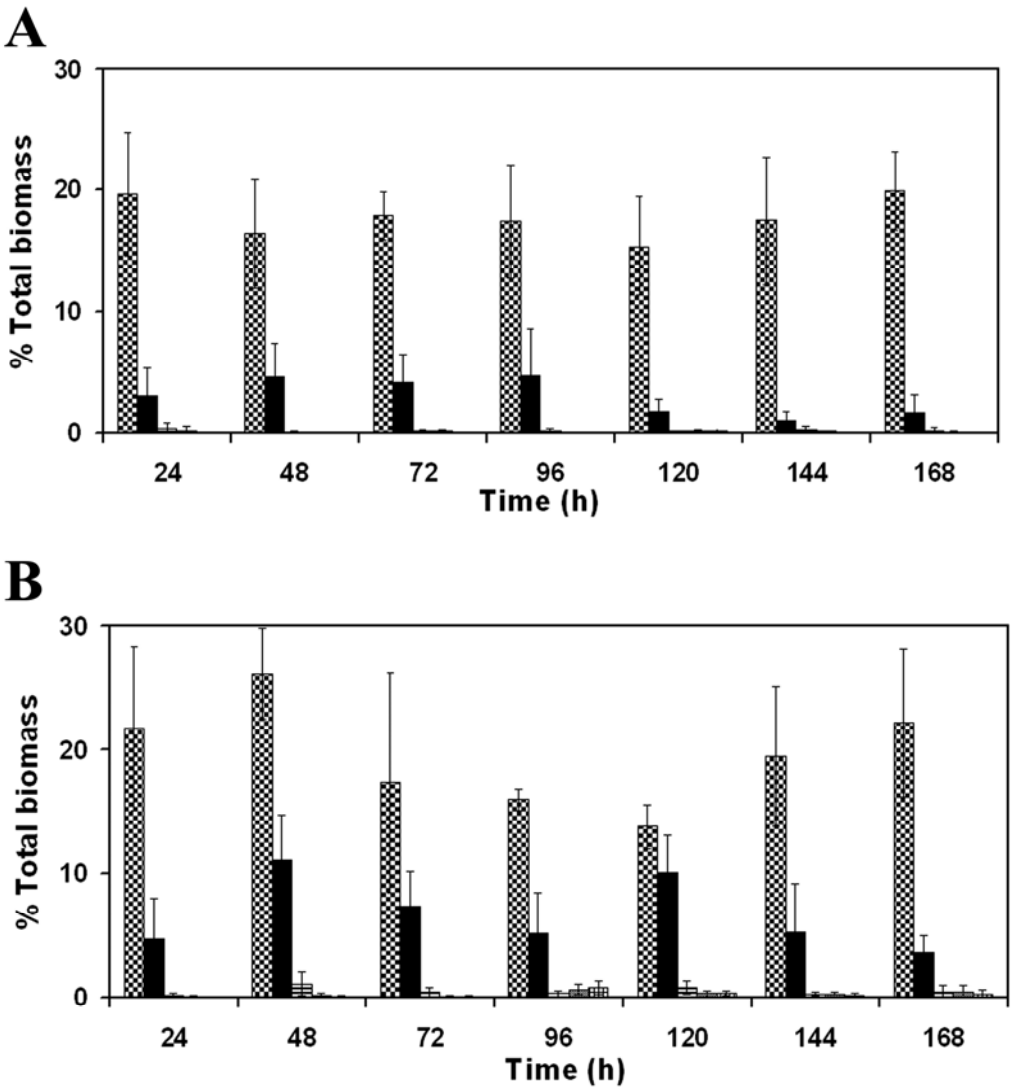

Figure 3.4.4. Abundance of total biomass of (A) low-flow and (B) high-flow biofilms over the 168 h growth period, as determined by fluorescein exclusion using CLSM and image analysis. The percent total biomass at each time interval and at each OTS depth is the average of 15 measurements made at random biofilm locations. The 0-µm OTS depth represents the biofilm-substratum interface. The symbols (▩), (■), (▤), (▩), and (▦) indicate the percent total biomass at OTS depths of 0, 3.7, 7.4, 11.1, and 14.8 µm, from left to right, respectively. The error bars indicate the standard deviation of the mean.

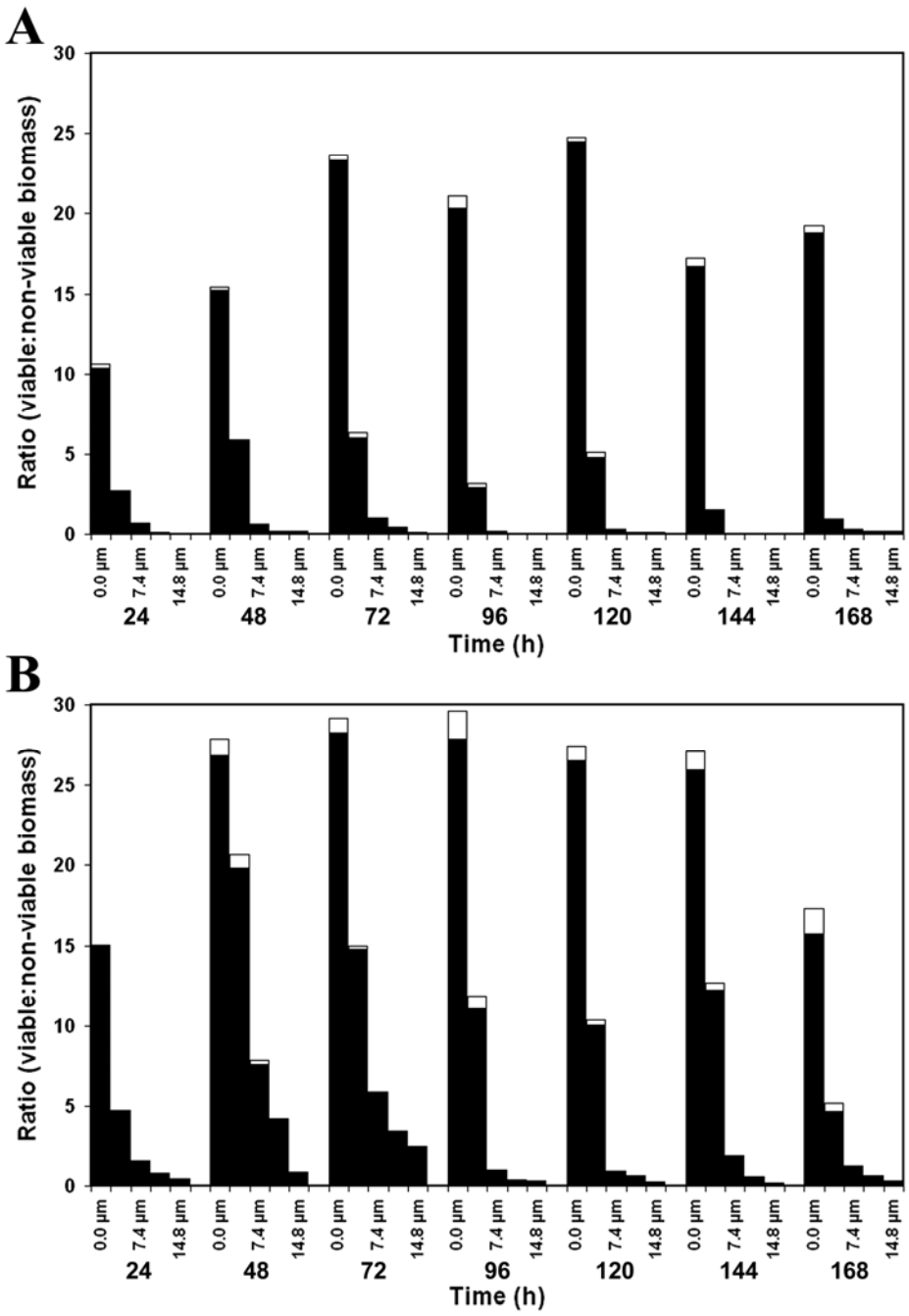

Figure 3.4.5. Viable biomass to non-viable biomass ratio in (A) low-flow and (B) high-flow biofilms over the 168 h growth period, as determined by staining with *Bac*Light™ Live/Dead Viability Probe and as estimated by CLSM and image analyses. The abundance of the viable and the non-viable biomasses for each time interval and at each OTS depth is the average of 15 measurements made at random biofilm locations. The 0-µm OTS depth represents the biofilm-substratum interface. The symbols (■) and (▭) indicate the abundance of viable and non-viable biomasses, respectively.

viable biomass in low-flow biofilms was determined to be 20:1, whereas, in high-flow biofilms the ratio was 14:1.

3.4.3. Influence of flow velocity on the glycoconjugate composition of biofilms

Lectin-binding analysis demonstrated that flow velocity significantly influenced the glycoconjugate composition of EPS of the biofilms (Figures 3.4.6 and 3.4.7; Table 3.4.1). The experiments showed that *Glycine max*-CY5, *Triticum vulgaris*-TRITC, and *Ulex europaeus*-FITC had the most extensive binding among the eight fluor-conjugated lectins analyzed for their binding to glycoconjugate residues of *Salmonella* serovar Enteritidis biofilms (Table 3.3.1). *N*-acetyl galactosamine (GalNAc) residues were detected by the binding of *Glycine max*-CY5 conjugate, *α*-L-fucose (fucose) residues were detected by the binding of *Ulex europaeus*-FITC conjugate, and *N*-acetyl glucosamine (GlcNAc2) and *N*-acetyl neuraminic acid (NeuNAc) residues in combination were detected by the binding of *Triticum vulgaris*-TRITC conjugate. Low-flow biofilms contained 55.2% GalNAc residues in their EPS; whereas, high-flow biofilms contained only 1.2% GalNAc residues. Conversely, low-flow biofilms contained 33.1% of fucose residues and 11.7% of GlcNAc2 and NeuNAc residues in combination (Table 3.4.1). However, high-flow biofilms contained a significantly higher ($P < 0.05$) concentration of these glycoconjugate components (69.1% and 29.6% of fucose residues, and GlcNAc2 and NeuNAc residues in combination, respectively).

3.4.4. Protein expression patterns of low-flow and high-flow biofilms

The proteome of 168 h low-flow and high-flow biofilms were compared with each other and that of planktonic cells continuously cultured for 168 h using 2D-PAGE. There were about 120 protein spots in the planktonic cell gel image; whereas, about 82 spots were identified in the biofilm gels. The majority of these proteins (48 spots) were found to become down-regulated when the cells were cultured as biofilms, relative to the planktonic cell control proteome. However, a few (20) proteins were found to be up-regulated. Overall, there was considerable similarity in the proteome of low-flow and high-flow biofilms; however, the proteomes of both biofilms were significantly different from that of the planktonic cell control proteome (Figure 3.4.8). There were 32

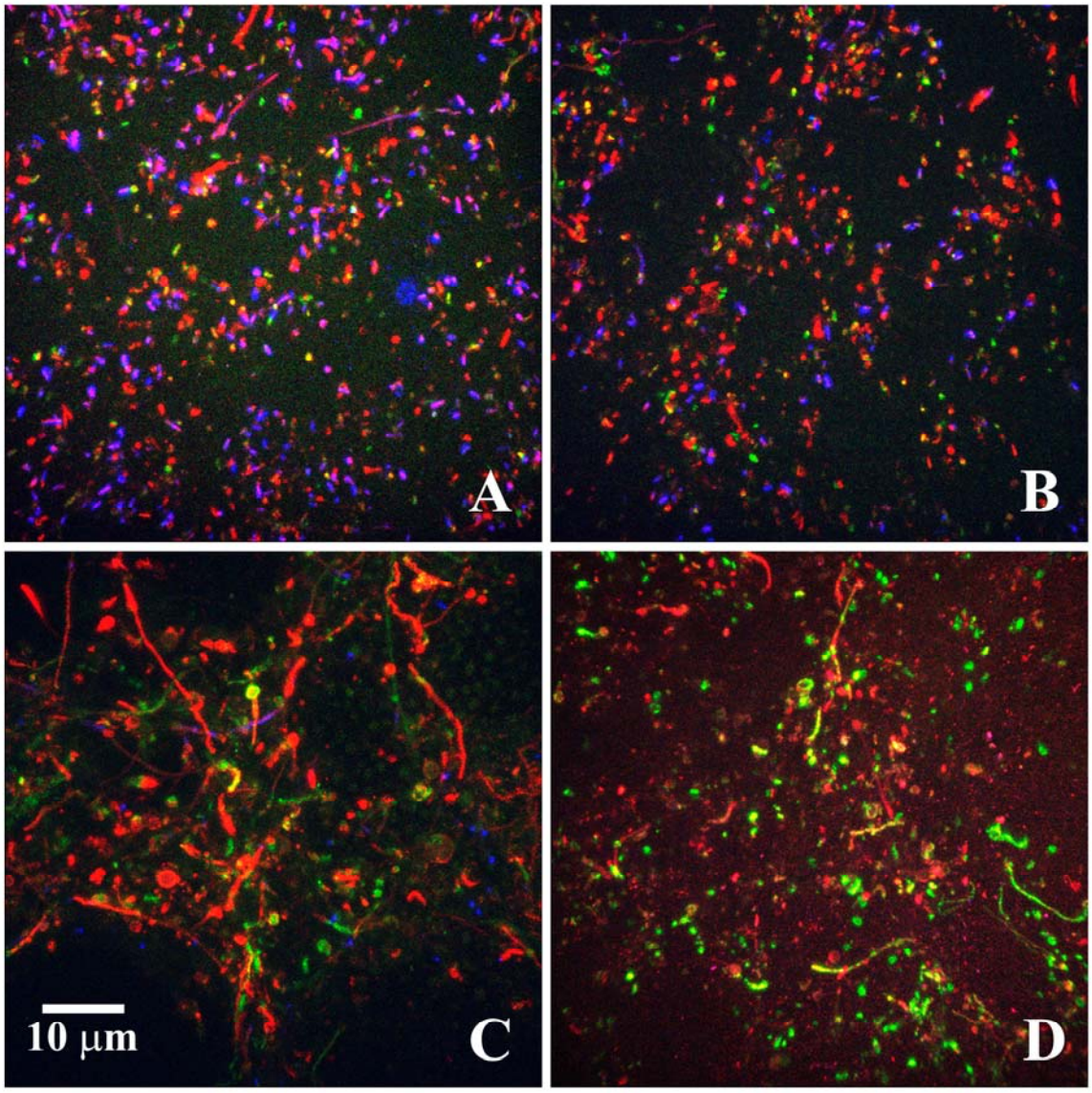

Figure 3.4.6. Representative projected micrographs of lectin-binding analyses, demonstrating the glycoconjugate composition of EPS in 168 h low-flow (A and B) and high-flow (C and D) biofilms of 0 to 15 μm and 0 to 45 μm biofilm thickness, respectively. The GalNAc residues of EPS are bound by *Glycine max*-CY5 conjugate (blue), *α*-L-fucose residues are bound by *Ulex europaeus*-FITC conjugate (green), and GlcNAc2 and NeuNAc residues are bound by *Triticum vulgaris*-TRITC conjugate (red). The italicized name indicates the lectin from the source plant, followed by the acronym indicating the labeled fluorescent dye.

Table 3.4.1. Glycoconjugate composition of EPS in 168 h low-flow and high-flow biofilms of *Salmonella* serovar Enteritidis, as determined by lectin-binding analyses

Glycoconjugates*	Proportion (%)	
	Low-flow biofilms	High-flow biofilms
N-acetyl galactosamine (GalNAc)	55.2	1.2
α-L-fucose	33.1	69.1
N-acetyl glucosamine (GlcNAc2) + *N*-acetyl neuraminic acid (NeuNAc)	11.7	29.7

* GalNAc residues are specifically bound by *Glycine max*-CY5 conjugate, *α*-L-fucose residues are specifically bound by *Ulex europaeus*-FITC conjugate, and GlcNAc2 + NeuNAc residues are specifically bound by *Triticum vulgaris*-TRITC conjugate. Italicized name indicates the lectin from the source plant, and the following acronym indicates the labeled fluorescent dye.

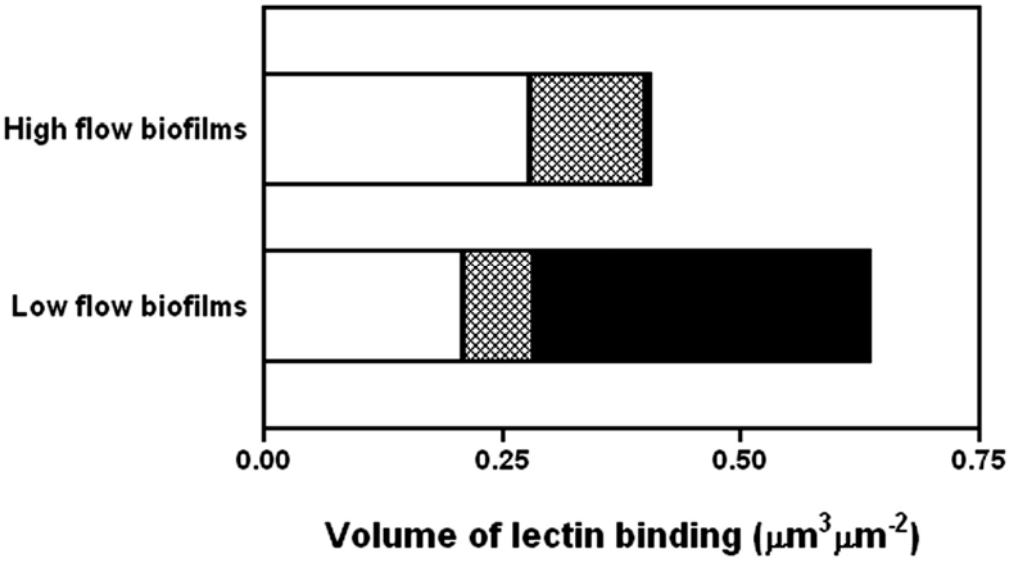

Figure 3.4.7. Proportional distribution of different glycoconjugates in the EPS of 168 h low-flow and high-flow biofilms. The symbols (▭), (▨), and (■) indicate the proportion of *α*-L-fucose, *N*-acetyl glucosamine (GlcNAc2) and *N*-acetyl neuraminic acid (NeuNAc) combination, and *N*-acetyl galactosamine (GalNAc), respectively.

differentially-expressed proteins by LC-MS/MS analysis (Tables 3.4.2 and 3.4.3). Among these proteins, 11 and 14 proteins were found to be up-regulated in low-flow and high-flow biofilms, respectively. However, 18 proteins were found to be down-regulated in both biofilm systems. Three proteins (GpmA, RpiA, and MalE), were found to be unchanged in their level of expression in low-flow biofilms, but became up-regulated in high-flow biofilms (Table 3.4.2). Major up-regulated proteins included those involved in degradation (PduA), energy metabolism (GapA, GpmA, Pgk, and RpiA), protein translation and modification (Tsf and TufA), RNA synthesis, RNA modification, and DNA transcription (RpoZ), cell processes (Crr, MalE, and PtsH) and adaptation (GrcA). Two hypothetical proteins, YcbL and YnaF, were also found to be up-regulated (Figure 3.4.8; Table 3.4.2). Major down-regulated proteins were those involved in degradation (GarR), proteolysis (DegQ), energy metabolism (AtpA, TpiA, Mdh, TalB, AceA, and AceK), cell envelope formation (RfbH, FljB, and FliC), nutrient-binding (ArgT, MglB, and OppA), detoxification (Tpx and SodB), chaperone protein Hsp70 (DnaK) and the hypothetical protein GntY (Figure 3.4.8; Table 3.4.3).

3.5. Discussion

In the present study, the timing and number of planktonic cells that released from the biofilms growing in low-flow and high-flow settings was used to assess when biofilms attained a pseudo-steady-state growth condition, and helped to define the extent of cell shedding from either glass or silicone tubing surfaces (materials both used in this flow cell model systems). The developmental kinetics of attached microorganisms has previously been shown to occur in a reproducible fashion, and cell dispersion (the release of cells from developing biofilms into the aqueous phase) has been used as a measurable parameter of biofilm development (Korber et al., 1989; Lawrence et al., 1997; Fux et al., 2004). *Salmonella* serovar Enteritidis biofilms invariably reached the pseudo-steady-state condition on either silicone or glass surfaces by ~96 h irrespective of flow velocity. While the factors that influence the timing of cell dispersion from biofilms are only beginning to be understood, both intrinsic genetic elements (*csrA* in *E. coli*, PP0164 and PP0165 genes in *P. putida*, etc.) and external environmental cues (glucose,

Figure 3.4.8. (facing page) Total proteins of p*I* values ranging from 4 and 7, which were differentially-expressed in 168 h low-flow and high-flow biofilms. The images illustrate the representative 2D-PAGE gels pertaining to proteins extracted from (A) planktonic cells cultured as continuous culture for 168 h (control), (B) low-flow biofilms, and (C) high-flow biofilms. The description of the proteins and their levels of expression are illustrated in Table 3.4.2 (up-regulation) and Table 3.4.3 (down-regulation). The symbol (□) indicate the location of the protein spot in the control and the protein spot which was unchanged in its level of expression in biofilms; the symbol (△) indicates up-regulation, and (▽) indicates down-regulation of the protein relative to the expression in the control.

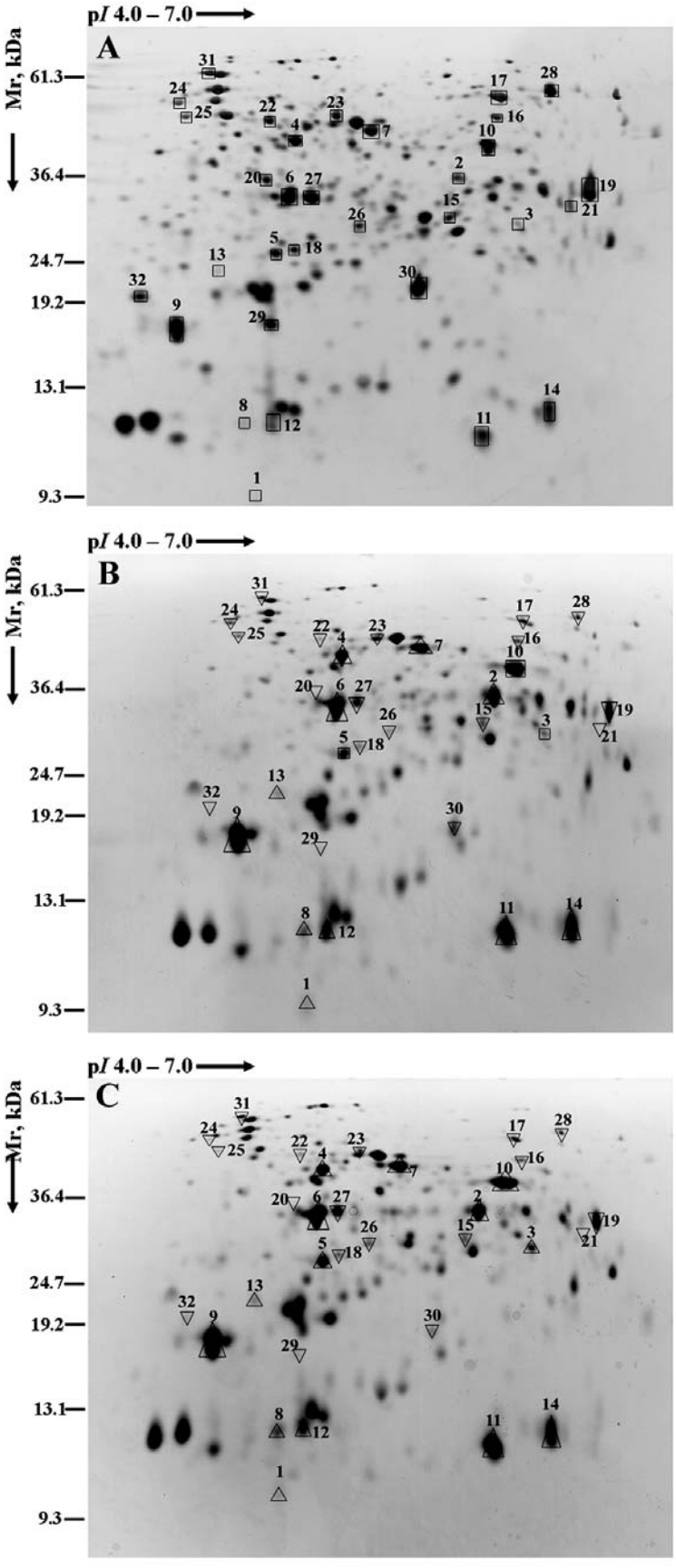
pI 4.0 – 7.0
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Table 3.4.2. Proteins up-regulated in 168 h *Salmonella* serovar Enteritidis biofilms

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold increase[b]	
						Low-flow biofilms	High-flow biofilms
Degradation (carbon compounds)	1	*pduA*	Putative propanediol utilization protein A	9.59	6.72	8.29	4.06
Energy metabolism (glycolysis)	2	*gapA*	Glyceraldehyde 3-phosphate dehydrogenase A	35.46	6.32	2.97	3.40
	3	*gpmA*	Phosphoglycerate mutase 1	28.36	5.78	0.95*	1.88
	4	*pgk*	Phosphoglycerate kinase	41.00	5.09	1.55	1.68
Energy metabolism (non-oxidative phase of pentose phosphate pathway)	5	*rpiA*	Ribose-5-phosphate isomerase A	22.90	5.08	1.17*	1.66
Protein translation and modification	6	*tsf*	Protein chain elongation factor Ts	30.36	5.13	1.67	2.02
	7	*tufA*	Translation elongation factor EF-Tu.A	43.40	5.30	1.56	1.88
RNA synthesis, RNA modification, and DNA transcription	8	*rpoZ*	DNA-directed RNA polymerase ω-chain	10.24	4.87	9.95	15.88
Cell processes (carbohydrate binding proteins)	9	*crr*	Phosphotransferase system enzyme II (Glucose-permease IIA component)	18.12	4.73	1.65	1.83
	10	*malE*	Maltose-binding periplasmic protein	43.18	6.27	1.33*	1.65
Cell processes (binding proteins [other])	11	*ptsH*	Phosphocarrier protein HPr	9.12	5.65	1.94	2.18
Adaptation and atypical conditions	12	*grcA*	Autonomous glycyl radical cofactor	14.34	5.10	2.84	3.13
Hypothetical proteins	13	*ycbL*	Hypothetical protein YcbL (putative metallo-*β*-lactamase)	23.73	4.95	4.16	5.17
	14	*ynaF*	Conserved hypothetical protein STY1416 (putative universal stress protein)	15.70	5.93	1.80	1.61

Mr, molecular mass; p*I*, isoelectric point.

[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.

[b] Fold increase in protein expression from that of continuously-cultured planktonic cells.

* Unchanged level of expression (i.e., 0.67 – 1.49).

Table 3.4.3. Proteins down-regulated in 168 h *Salmonella* serovar Enteritidis biofilms

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold decrease[b]	
						Low-flow biofilms	High-flow biofilms
Degradation (carbon compounds)	15	*garR*	Tartronate semialdehyde reductase	30.73	5.59	0.53	0.48
Degradation (proteins, peptides, and glycoproteins)	16	*degQ*	Serine endoprotease	47.28	6.80	0.53	0.51
Energy metabolism (ATP synthesis)	17	*atpA*	ATP synthase α-subunit	54.98	5.80	0.24	0.20
Energy metabolism (glycolysis)	18	*tpiA*	Triose-phosphate isomerase	26.92	5.68	0.45	0.64
Energy metabolism (tricarboxylic acid cycle)	19	*mdh*	Malate dehydrogenase	32.48	6.01	0.41	0.53
Energy metabolism (non-oxidative phase of pentose phosphate pathway)	20	*talB*	Transaldolase B	35.04	5.09	0.21	0.30
Energy metabolism (glyoxylate bypass)	21	*aceA*	Isocitrate lyase	47.56	5.22	0.13	0.20
	22	*aceK*	Isocitrate dehydrogenase kinase/phosphatase	46.09	5.99	0.05	0.13
Cell envelope (lipopolysaccharides)	23	*rfbH*	Lipopolysaccharide biosynthesis protein	48.10	5.27	0.45	0.46
Cell envelope (surface structures)	24	*fljB*	Phase 2 flagellin	52.41	4.75	0.54	0.31
	25	*fliC*	Phase 1 flagellin	51.48	4.79	0.49	0.10

Continued…

Table 3.4.3. continued.

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold decrease[b]	
						Low-flow biofilms	High-flow biofilms
Cell processes (aminoacid and amine binding proteins)	26	*argT*	Lysine-arginine-ornithine-binding periplasmic protein	28.20	5.99	0.25	0.50
Cell processes (carbohydrate binding proteins)	27	*mglB*	D-galactose/D-glucose-binding periplasmic protein	35.81	5.81	0.35	0.48
Cell processes (binding proteins [other])	28	*oppA*	Oligo-peptide binding protein complexed with Kvk, chain A	58.81	5.85	0.11	0.19
Cell processes (detoxification)	29	*tpx*	Thiol peroxidase	17.98	4.75	0.24	0.43
	30	*sodB*	Superoxide dismutase [Fe]	21.18	5.58	0.21	0.05
Cell processes (chaperones)	31	*dnaK*	Chaperone protein Hsp70	69.13	4.83	0.20	0.24
Hypothetical proteins	32	*gntY*	Hypothetical protein GntY	20.94	4.52	0.03	0.03

Mr, molecular mass; p*I*, isoelectric point.
[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.
[b] Fold decrease in protein expression from that of continuously-cultured planktonic cells.

nitrogen and phosphorus availability, O_2 depletion, shear stress, etc.) have been shown to be involved (Jackson et al., 2002; Gjermansen et al., 2005).

The formation of microcolonies was a common feature of low-flow and high-flow biofilms during the early stages (until 48 h) of development, as reported previously (Purevdorj et al., 2002). These microcolonies ultimately merged as the biofilms matured, but were still evident as bacterial mounds separated by voids or water channels. At steady-state (~96 h), *Salmonella* serovar Enteritidis biofilms exhibited the architecture typical of monospecies biofilms, with cell aggregates or mounds interlaced with exopolymer and surrounded by open areas or channels where nutrient medium flowed (Figure 3.4.2) (Lawrence et al., 1991; Davey and O'Toole, 2000). The thicknesses of *Salmonella* serovar Enteritidis biofilms thus varied considerably with location (whether the random thickness measurement occurred within a mound or in the separating water channel between mounds), and also had high thickness measurement standard deviations (Figure 3.4.2). Parameters such as average biofilm thickness have been used to measure the impact of mutations in genes involved in motility, cell signaling, and stationary phase σ-factor on *P. aeruginosa* biofilm development (Heydorn et al., 2002). The standard deviation of thickness measurements was directly proportional to the corresponding mean thickness values within the range examined (*Re* for low-flow and high-flow conditions was 0.237 and 2.37, respectively), indicating that thicker biofilms had larger mounds and deeper channels. It is thought that the considerable variation in the biofilm thickness of high-flow biofilms were the cumulative consequence of growth, viscoelasticity, sloughing (shedding), and re-growth of biofilm material, which together contributed to the pseudo-steady-state condition of the biofilms. These observations are similar to reports of *Sphingomonas* spp. biofilms grown in laminar flow conditions, where microcolonies and extensive water channels developed and the microcolonies moved over the attachment surface as a unit under the influence of nutrient flow (Venugopalan et al., 2005).

During the initial 24 h of *Salmonella* serovar Enteritidis biofilm development, there was no significant effect of nutrient flow velocity on conformation and distribution of microcolonies (Figure 3.4.2A and B). However, the average maximum width of water channels in high-flow *Salmonella* serovar Enteritidis biofilms increased during the

course of biofilm development. In contrast, channel width decreased due to increased EPS production in *Sphingomonas* biofilms (Venugopalan et al., 2005). Exopolymers played a significant role in the architecture of *Salmonella* serovar Enteritidis biofilms, as the biofilms grown under high-flow conditions were subject to visible flow peristalsis-induced viscoelasticity. It has been reported that biofilm viscoelasticity allows the biofilms to structurally deform when exposed to varying shear stresses (Stoodley et al., 2000), a mechanism that allows the biofilms to resist erosion due to increased fluid shear by deformation while remaining attached to the surface (Rupp et al., 2005). It is accepted that channel formation improves the diffusion-dominated transport of nutrients and waste materials into and out of the biofilm matrix; therefore, it is envisaged that the *Salmonella* serovar Enteritidis biofilms are highly polymorphic and structurally-adapted to changes in substratum chemistry, flow velocity and thus nutrient availability and waste removal.

There are reports that the major components of the EPS of *S. enterica* biofilms are cellulose and colonic acid (Solano et al., 2002; Prouty and Gunn, 2003; Ledeboer and Jones, 2005). The identification of chemical heterogeneities spatially-localized within biofilms using fluor-conjugated lectins (Neu and Lawrence, 1999; Neu et al., 2001; Laue et al., 2006) could help explain the various roles of EPS in these systems. In this study, nutrient flow velocity was implicated in a shift in glycoconjugate composition of *Salmonella* serovar Enteritidis biofilms; flow velocity apparently induced high-flow biofilms to alter their extracellular glycoconjugates as an adaptive mechanism to enhanced shear stress, possibly favoring deformation. The major glycoconjugate component of the EPS of low-flow biofilms was found to be GalNAc residues; whereas, in high-flow biofilms the major component detected was fucose. Changes in EPS composition of biofilms in response to various conditions, including nutrients, pH, temperature, growth medium and substratum, and developmental time have been reported (Lee et al., 1997; Tavernier et al., 1997; Lawrence et al., 2004, 2005).

Combining the qualitative and quantitative information, it was concluded that high-flow biofilms were more compact over the 0 to 15 μm OTS depths than were the low-flow biofilms (Figures 3.4.4 and 3.4.5). In general, low-flow biofilms were found to consist of single cells arranged rigidly in compact masses (Figure 3.4.6A and B);

whereas, high-flow biofilm cells were loosely arranged and included long chains or filaments (Figure 3.4.6C and D). These elongated chains of cells may represent phenotypic variants due to the decreased O_2 or nutrients, as well as responses to other stress factors (Korber et al., 1994), and may also suggest that the metabolism of biofilm bacteria had shifted between flow regimens.

Differential protein expression of planktonic and biofilm modes of growth has been reported in *E. coli* O157:H7 (Trémoulet et al., 2002), *P. putida* (Sauer and Camper, 2001), *Bacillus cereus* (Oosthuizen et al., 2002), and *S. aureus* (Resch et al., 2005). In this study, out of the 14 proteins up-regulated in high-flow biofilms (relative to the planktonic cell control), 11 were also up-regulated in their levels of expression in low-flow biofilms, and three proteins (GpmA, RpiA, and MalE) were up-regulated in the high-flow biofilms only. However, all 18 proteins down-regulated in high-flow biofilms were found to be down-regulated in low-flow biofilms. Overall, there was significant similarity in the protein expression profiles of both low-flow and high-flow biofilm systems, with the exception of the expression levels of GpmA, RpiA, and MalE. In *S. enterica*, PduA was suggested to be involved in 1,2-propanediol (1,2-PD) utilization to allow use of this compound as a carbon source for energy generation (Jeter and Roth, 1987). Interestingly, the up-regulation of PduA observed in low-flow and high-flow biofilms suggests that these cells utilized the 1,2-PD utilization pathway perhaps for energy generation, minimizing aldehyde toxicity, or for cobalamin biosynthesis.

Proteins involved in degradation of carbon compounds (GarR) and proteins (DegQ) were significantly down-regulated in both biofilm systems relative to the planktonic cell control proteome. Similarly, proteases were found to be up-regulated in planktonic *S. aureus* cells in comparison with the biofilm cells (Resch et al., 2005). Among the proteins involved in energy generation, GapA, GpmA, Pgk, and RpiA were significantly up-regulated in the present study; whereas, AtpA, TpiA, Mdh, and TalB were found to be down-regulated. Interestingly, malate dehydrogenase (Mdh) has been found to be up-regulated in *E. coli* O157:H7 biofilms (Trémoulet et al., 2002). Two enzymes involved in glyoxylate bypass, AceA and AceK, were found to be down-regulated in both low-flow and high-flow biofilms. The down-regulation of both AceA and AceK indicate that both low-flow and high-flow biofilms did not employ glyoxylate

bypass during acetate metabolism for energy generation (Chung et al., 1988). Some proteins involved in nutrient-binding and transport, namely, Crr (glucose), MalE (maltose), PtsH (various sugars) were found to be up-regulated. ArgT (lysine, arginine, and ornithine), MglB (galactose and glucose), and OppA (oligo-peptides) were found to be down-regulated. There are reports that D-galactose binding protein and amino acid ABC transporter-binding protein were up-regulated in *E. coli* O157:H7 biofilms (Trémoulet et al., 2002).

Increased protein biosynthesis in both biofilm systems was deduced from the up-regulation of translation elongation factors (TufA and Tsf) and a protein involved in DNA transcription and RNA synthesis (RpoZ). The translational elongation factor G (Fus) has recently been reported to be up-regulated in *S. aureus* biofilms (Resch et al., 2005), as were translational elongation factors in *L. monocytogenes* biofilms (Hefford et al., 2005). However, proteins involved in synthesis of structural components of the cell envelope, namely, lipopolysaccharide biosynthesis protein (RfbH) and flagellar biosynthesis proteins (FljB and FliC), were found to be down-regulated in biofilms during the present study. There are similar reports that FliC was expressed in planktonic cells of *P. putida* but not in biofilms; however, two different lipopolysaccharide biosynthesis proteins, LpxD and WbpG appeared to be up-regulated in *P. putida* biofilms (Sauer and Camper, 2001).

The patterns of up-regulation and down-regulation of proteins involved in adaptation to stress conditions were similar in low-flow and high-flow biofilms. GrcA, which acts as a radical domain for damaged pyruvate/formate lyase was significantly up-regulated. However, chaperone protein Hsp70 (DnaK), which is involved in heat-shock and similar stress response conditions, and SodB and Tpx involved in survival of oxidative stress, were significantly down-regulated in both biofilm systems. Thus, it is envisaged that low-flow and high-flow biofilms experienced more or less similar stress conditions, something one would expect if structural changes to the biofilm were responses that served to minimize overall stress. In the case of high-flow biofilms, the increased shear stress might have been compensated by the shift in extracellular glycoconjugate composition and the increased mass transfer of nutrients into the biofilms and removal of waste materials brought about by the architectural adaptation

(i.e., formation of voids and mounds). The protein expression patterns of *Salmonella* serovar Enteritidis biofilms appeared to be unique relative to planktonic cells and presumed to be significantly influenced by the nutrient composition and other environmental or stress factors, however flow velocity at the rates tested did not have a significant effect.

In conclusion, a 10-fold difference in nutrient laminar flow velocity significantly influenced the development and architecture of *Salmonella* serovar Enteritidis biofilms. However, the influence of nutrient concentration and nutrient composition in the biofilm development and architecture has not been examined in this study. The thickness of *Salmonella* serovar Enteritidis biofilms was related to the velocity of nutrient laminar flow, and biofilms grown under low-flow conditions contained a higher volume of EPS than high-flow biofilms; the composition of glycoconjugates detected was also distinct. The protein expression patterns of the biofilms were essentially similar, indicating that the distinct biofilm architectures and patterns of EPS composition exhibited under the different flow regimens was a physical consequence of flow, but did not involve major shifts in protein expression (at least not at the point of steady-state equilibrium). These findings suggest that complex mechanisms are operational that result in modification of extracellular glycoconjugates that are not dependent upon large scale shifts in protein expression, or that chemical evidence of this event was simply not seen since proteomic analysis was only performed at 168 h. These results are also supportive of the notion that biofilms are automatonic systems that respond to physical and chemical conditions such as flow rate and nutrients, with the end result being different biofilm architectures that optimize conditions (Van Loosdrecht et al., 1997; Wimpenny and Colasanti, 1997). While the proteomes of biofilms cultivated under low-flow and high-flow conditions were not significantly different, the biofilm protein expression patterns were significantly different from that of planktonic *Salmonella* serovar Enteritidis cells. It is suggested that the *Salmonella* serovar Enteritidis biofilms were structurally-adapted to changes in substratum chemistry, flow velocity and thus nutrient availability and waste removal. This might have significance in controlling biofilms on food processing surfaces, as hydrodynamic conditions generated by mechanical processes like routine scrubbing have limited and short-term effects. The biofilms subsequently re-grow and

become structurally-adapted to compensate for adverse effects, within hours after the application of stressors. The survival and persistence of pathogens in these environments depend largely on the ability of the biofilms to minimize overall stress and to re-grow and adapt following hydrodynamic and mechanical disturbances.

3.6. Connection to the next study

Nutrient flow velocity influences biofilm thickness; high-flow biofilms are thicker than the low-flow biofilms. Thus, the next study was performed to elucidate how flow velocity influences biofilm shearability, as well as morphology and physiology of cells in different biofilm locations.

4. CELLS IN SHEARABLE AND NON-SHEARABLE REGIONS OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS BIOFILMS ARE MORPHOLOGICALLY AND PHYSIOLOGICALLY DISTINCT

4.1. Abstract

The cellular morphology, exopolymer chemistry, and protein expression of shearable and non-shearable fractions of *Salmonella enterica* serovar Enteritidis biofilms were examined. The biofilms were grown at a nutrient laminar flow velocity of 0.07 cm sec^{-1} for ~120 h, resulting in biofilms with a thickness (mean ± standard deviation) of 43 ± 24 μm. An empirically-determined shear-inducing flow (1.33 cm sec^{-1}) was then applied for 5 min, effectively reducing the biofilm thickness by approximately 70%, leaving 13 ± 6 μm of non-shearable material. There was no significant difference in the exopolymer glycoconjugate composition of shearable and non-shearable biofilm regions. Length:width indices (LWI) of the cells in non-shearable and shearable biofilm regions, as well as planktonic cells from biofilm effluent and continuous culture were determined to be 3.2, 2.3, 2.2, and 1.7, respectively, indicating that the cells in the shearable biofilm fraction were morphologically more similar to planktonic cells than to the cells in the non-shearable biofilm fraction. Seven proteins (CspA, Eno, GrcA, Hns, Tsf, Tuf, and YjgF) were expressed at relatively higher levels in the cells of shearable fraction;

This chapter is reproduced from a manuscript prepared for publication. The manuscript is co-authored by J. R. Lawrence and D. R. Korber. All experimental works with the exception of confocal laser microscopy data analyses and mass spectrometry analyses were carried out by me. In addition, I wrote the initial draft of the manuscript. Minor modifications were made to the prepared version to maintain thesis format and style.

whereas, eight proteins (AtpA, DegQ, DnaK, GpmA, OppA, PduA, TpiA, and WrbA) were expressed at relatively higher levels in the cells of non-shearable fraction. Enhanced expression of proteins involved in cold-shock response, adaptation, and broad regulatory functions (CspA, GrcA, and Hns, respectively) in the shearable region, and enhanced expression of protein involved in heat-shock response and chaperonin function (DnaK) in the non-shearable region indicated that the physiological status of cells in the two biofilm regions was distinct. Differential cellular morphological and physiological changes in the two biofilm regions are suggested to be adaptive responses to various stressors prevailing in the microenvironments.

4.2. Introduction

Biofilms constitute a protected mode of growth that allows survival in a hostile environment, where the slow growth of cells in combination with spatial and chemical heterogeneity are thought to contribute to biofilm resistance to various control strategies (antibiotics and other antimicrobial agents) (Costerton et al., 1999) as well as the formation of "persisters" within the biofilms (Lewis, 2001). Hydrodynamic conditions are known to influence overall biofilm development and morphology by controlling two interlinked parameters, namely, mass transfer and drag (Stoodley et al., 1998). Lewandowski and Walser (1991) reported that the thickness of heterogeneous biofilms is at a maximum near the transition between laminar and turbulent flows, and hypothesized that an optimal Reynolds number existed below which biofilm biomass accumulation was mass transfer-limited and above which accumulation was limited by shear-induced biofilm detachment (Lewandowski and Walser, 1991; Stoodley et al., 1998, 1999).

There exist distinct chemical zones (or gradients) within thicker biofilms that result in different sets of genes and proteins being expressed; some of these chemical and physiological differences may indeed cause, or contribute to, cell shedding events (Donlan, 2002; Sauer et al., 2002; Werner et al., 2004). Differential alginate gene (*algC*) expression has been reported in *P. aeruginosa* biofilm cells, which could be attributed to age, nutritional status, or nature of adherence of the cells to the substratum (Davies et al., 1993). Activation of the *algC* promoter was observed only in 30% of the cells adhering to the surface, indicating the variability of expression of alginate genes among biofilm

cells. Similarly, stratified patterns of protein synthesis and growth have been seen in *P. aeruginosa* biofilms; actively growing cells were localized near the air interface of biofilms whereas the cells in the remainder of the biofilm were relatively inactive (Werner et al., 2004).

Cellular morphological changes such as minicell or filament formation, or the appearance or disappearance of cellular surface structures (e.g., motility apparatus) have also been reported in bacteria (Jacobs and Shapiro, 1999; Steinberger et al., 2002). Most of the morphological changes observed in *E. coli* and *S. enterica* may be associated with the effects of environmental stressors (Shaw, 1968; Philips et al., 1998; Mattick et al., 2000, Steinberger et al., 2002). In *P. aeruginosa* biofilms growing in nutrient-limiting environments, cells increased their surface area to volume ratio by elongating, thereby morphologically adapting to starvation; whereas, the adaptive response to starvation in planktonic cells grown in well-mixed environments involved increasing the ratio of surface area to volume by reducing cell size (Steinberger et al., 2002). Cellular morphological changes in bacteria have also been regarded as an important adaptive mechanism to various environmental stressors.

Once a biofilm is formed and the exopolymer (EPS) matrix has been secreted by the sessile cells, the resultant structure is highly viscoelastic (Stoodley et al., 1998). The EPS slime matrix determines the cohesive strength of bacterial biofilms and provides protection to biofilm cells (Stoodley et al., 2002). It was reported that viscoelasticity allowed *S. aureus* biofilms to resist detachment due to increased fluid shear by becoming deformed while remaining attached to the surface (Rupp et al., 2005). When biofilms are formed in low-shear environments, they have a low tensile strength and break easily, whereas biofilms formed in high-shear environments are remarkably strong and resistant to mechanical breakage (Donlan and Costerton, 2002).

There are no reports on the use of shearability and detachment characteristics for the analysis of physiological regions in *Salmonella* biofilms. The concept of fractionating biofilms, illustrated in Figure 4.2.1, was used in conjunction with *Salmonella* serovar Enteritidis, an important foodborne enteric pathogen to examine whether the biofilm cells in the shearable and non-shearable regions of biofilms growing in a laminar flow environment were physiologically distinct. The following approaches

were therefore employed: (i) bright-field microscopy to assess the shearability of biofilms, (ii) confocal laser scanning microscopy (CLSM) to analyze the EPS composition (iii) atomic force microscopy (AFM) to analyze cellular morphological features, and (iv) two dimensional polyacrylamide gel electrophoresis (2D-PAGE) to analyze differential protein expression.

4.3. Materials and methods

4.3.1. Media and chemicals

Tryptic Soy Agar (TSA) and Trypticase Soy Broth (TSB) were purchased from BBL (Becton Dickinson, Cockeysville, MD); magnesium chloride ($MgCl_2$), phenylmethylsulphonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), DNase, RNase A, bromophenol blue, DL-dithiothreitol (DTT), and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO); sodium chloride (NaCl) was from EM Science (Gibbstown, NJ); EDTA was from J. T. Baker Chemical Co. (Philipsburg, NJ); glycerol, sodium dodecyl sulphate (SDS), Tris base, and urea were purchased from Life Technologies (Grand Island, NY); and immobilized pH gradient buffer (pH 4.0 to 7.0), Immobiline DryStrip gels, and PlusOne^{TM} protein silver staining kit were purchased from GE Healthcare Bio-Sciences Inc. (Baie d'Urfé, QC, Canada).

4.3.2. Bacteria and culture conditions

Prior to the experiment, *Salmonella enterica* serovar Enteritidis ATCC 4931 (referred to as *Salmonella* serovar Enteritidis) was cultured from a frozen stock on TSA plates overnight at 37ºC. Cells in the mid-log phase of growth were obtained by transferring a loopful of colony material from TSA plates to 50 ml of 10% [wt/vol] TSB in an Erlenmeyer flask and incubating on a gyratory shaker (150 ± 5 rpm) at room temperature (RT; 21 ± 2ºC) in batch culture for approximately 12 h. These cells, which were previously determined to be in the mid-log phase of growth via growth curve experiment, were used to inoculate flow cells. Planktonic cells for control experiments were grown in continuous culture in an Erlenmeyer flask held on a gyratory shaker (150

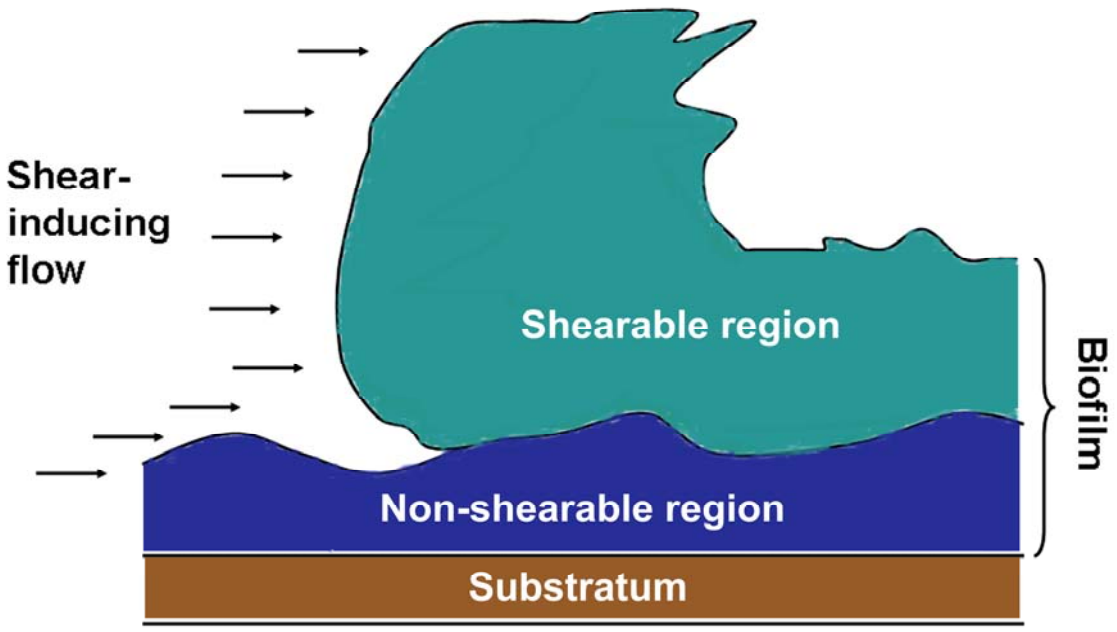


Figure 4.2.1. Conceptual illustration of biofilm shearing into shearable and non-shearable biofilm regions by means of shear-inducing flow.

± 5 rpm) and incubated at RT for 120 h; the total culture volume was set at 175 ml. Nutrient medium (10% [wt/vol] TSB) was added and removed continuously at 25 ml h^{-1} resulting in a dilution rate of 0.14 h^{-1}. The medium was pumped into and out of the Erlenmeyer flask via silicon tubing using two Watson-Marlow peristaltic pumps (Model 202U; Watson-Marlow, Cornwall, UK), with each one set to control the input of the medium to the flask and removal of the effluent to a waste reservoir.

4.3.3. Flow cells, inoculation, and flow velocity

Multi-channel flow cells were constructed using 5 mm sheets of polycarbonate plastic into which were milled channels that were then covered with glass coverslips, as described previously (Korber et al., 1994). Flow cell channels were sterilized by flushing with 5.25% [wt/vol] sodium hypochlorite solution for a period of 10 min. Reservoirs of sterile nutrient medium (10% [wt/vol] TSB) were connected via silicon tubing to the flow cell channels and subsequently connected to the effluent reservoir. The medium was pumped through flow cells using a Watson-Marlow peristaltic pump. Each flow cell channel was separately inoculated with 0.5 ml mid-log phase *Salmonella* serovar Enteritidis cells, prepared as outlined above, concentrated or diluted to an optical density equivalent to 0.5 McFarland standard (1.5 X 10^8 cfu ml^{-1}). Following injection, the inoculum was retained in the flow cell for 15 min to facilitate cell adhesion to flow cell channel surfaces. Bulk flow rates of 2.5 ml h^{-1} and 25 ml h^{-1} resulted in laminar flow velocities of 0.007 cm sec^{-1} and 0.07 cm sec^{-1}, respectively. Biofilms grown at nutrient laminar flow velocities of 0.007 cm sec^{-1} and 0.07 cm sec^{-1} will hereafter known as low-flow and high-flow biofilms, respectively.

4.3.4. Shear-inducing flow

Salmonella serovar Enteritidis biofilms were grown until "steady-state condition" (~120 h), which was defined based on patterns of cell shedding and biofilm thickness under low-flow and high-flow regimens. The biofilms were exposed to an empirically-determined high-rate flow pulse (hereafter referred to as shear-inducing flow; 1.33 cm sec^{-1}, ~20 times greater than the 0.07 cm sec^{-1} flow condition). This flow rate was chosen based on its ability to fractionate low-flow and high-flow biofilms into two separate

zones. Shear-inducing flow represents a fluid flow velocity that removes or "shears" loosely attached cell material (shearable portion) from the biofilms, leaving a more firmly-attached material (non-shearable portion) on the flow cell surface. The effect of shear-inducing flow on the low-flow and high-flow biofilms was studied by growing the biofilms in two separate flow cells until steady-state was reached. Shear-inducing flow rate was then applied for 5 min, and the extent of separation of the shearable portion in each case quantified by measuring the thickness (*see* below) of the biofilms at different random locations before and after shearing. Cells from shearable and non-shearable biofilm regions as well as biofilm effluent were collected from independently-grown high-flow biofilms.

4.3.5. Biofilm thickness measurements

The thickness of biofilms grown at the two different flow velocities, before and after the application of shear-inducing flow was measured in micrometers using a Nikon Microphot-FXA microscope (Nikon Corp., Tokyo, Japan) (Korber et al., 1994). Fifteen random fields were assessed for each biofilm with five separate thickness values obtained per field (n = 75). These values were averaged to obtain the thickness of each biofilm. Each thickness measurement was the average of 225 thickness measurements made at random locations from three experimentally-replicated biofilms.

4.3.6. Determination of EPS glycoconjugate composition

Application of a panel of fluor-lectin conjugates showed that *Glycine max*-CY5, *Triticum vulgaris*-TRITC, and *Ulex europaeus*-FITC had extensive binding to glycoconjugate residues of *Salmonella* serovar Enteritidis biofilms. The fluor-conjugated lectins *Glycine max*-CY5, *Triticum vulgaris*-TRITC, and *Ulex europaeus*-FITC were used in combination (triple lectin labeling) for *in situ* analyses of EPS composition (Neu et al., 2001). *N*-acetyl galactosamine (GalNAc) residues in the EPS were detected by the binding of the *Glycine max*-CY5 conjugate, α-L-fucose (fucose) residues were detected by the binding of the *Ulex europaeus*-FITC conjugate, and *N*-acetyl glucosamine (GlcNAc2) and *N*-acetyl neuraminic acid (NeuNAc) residues were detected by the binding of the *Triticum vulgaris*-TRITC conjugate. Analysis of the binding of

fluorescent lectins was carried out using a Bio-Rad MRC-1024 confocal laser scanning microscope mounted on a Nikon Microphot-SA microscope. CLSM optical thin sections (OTSs) were collected in each of the three channels to determine the EPS biomass and composition at various depths (n = 15). Digital image analyses and calculations were performed using NIH Image version 1.61 with macros written for semi-automated quantification, as described previously (Lawrence et al., 1998; Neu and Lawrence, 1999; Neu et al., 2001). Three color (red-green-blue) projections of the biofilms were prepared using Adobe Photoshop version 7.0.

4.3.7. Determination of cellular dimensions using AFM

Cellular dimensions (length (*l*), width (*w*), and depth (*d*)) of 120 h high-flow biofilm cells (from shearable and non-shearable regions) and planktonic cells (from biofilm effluent and continuous culture) were determined using AFM and image analyses. Planktonic and biofilm cells from the shearable biofilm region were allowed to air-dry on a glass coverslip for ~15 min, washed with phosphate buffered saline (pH 7.2), rinsed with sterile reverse osmosis (RO) water. Non-shearable biofilm cells were prepared for AFM by removing pieces of coverslips from the flow cell channels and affixing the pieces onto a mounting coverslip using silicon adhesive. Subsequent washing was performed just as in the case of planktonic cells. The samples were allowed to air-dry for ~15 min and placed in a humidifying chamber (RT and ~80% rH) until the microscopy was performed (Cross et al., 2006; Del Sol et al., 2007).

AFM was conducted using a Molecular Imaging PicoSPM I^{TM} with PicoScan 2100^{TM} controller and equipped with a piezo-scanner (Molecular Imaging Corp., Tempe, AZ). Imaging was carried out using oxidation-sharpened silicon nitride AFM probes with a tip radius of ca. 10 nm (cantilever nominal spring constant, k = 0.12 N m^{-1}; resonant frequency, 14 – 26 kHz) (DNP-S; Veeco Probes, Camarillo, CA) in the contact mode. The scanning force constant was ca. 3 nN and scan rates were ca. 1 Hz. Image areas of 10 X 10 and 20 X 20 μm^2 were collected from the same location in height and deflection modes simultaneously (Touhami et al., 2004; Pelling et al., 2005; Cross et al., 2006). Quantitative data were collected from the height mode images, whereas corresponding images for illustration purpose were collected in the deflection mode.

Images were collected from samples derived from three independently replicated experiments. The stored images were analyzed using PicoScan™ software version 5.3.1a (Molecular Imaging Corp.) for determining *l*, *w*, and *d* of randomly selected cells (n = 80). Similar analyses for determining *l* and *d* have been described previously (Steinberger et al., 2002). Length:width indices (LWI) of the cells from each region was calculated by dividing the respective average cell length value with the corresponding average cell width value. The cells were mathematically modeled as rectangular boxes and thus, the cell surface area (SA) and cellular volume (V) were calculated using the following equations, as reported by Steinberger et al. (2002):

$$SA = (2l \,.\, w) + (2l \,.\, d) + (2w \,.\, d) \quad (4.1)$$

$$V = l \,.\, w. \, d \quad (4.2)$$

where, *l* is cellular length, *w* is cellular width, and *d* is cellular depth.

4.3.8. Protein expression analyses and protein identification

High-flow biofilms grown for 120 h were separated into shearable and non-shearable portions by the application of the shear-inducing force as described above. The shearable portion was collected in a sterile centrifuge tube and the non-shearable portion was subsequently scraped from the flow cell channels. The biofilm cells were pelleted by centrifuging at 4,000 rpm (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 5 min. Total cellular proteins from each biofilm fraction were separated by 2D-PAGE, as detailed previously (Mangalappalli-Illathu and Korber, 2006). Differentially-expressed proteins were detected and quantified from the stored images of gels using Phoretix™ 2D version 2004 analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Protein spot volumes from each biofilm fraction were assessed by averaging spot volumes from three 2D-PAGE gels replicated experimentally. Three gels each, representing proteins from shearable and non-shearable regions of high-flow biofilms were averaged and used as control to determine the differential expression of proteins. An enhanced level of expression (1.5-fold or more) was interpreted as an increase, whereas, a reduced level of expression (1.5-fold or more) was interpreted as a decrease in protein expression. Differentially-expressed proteins were identified by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

analysis, as reported previously (Mangalappalli-Illathu and Korber, 2006). The LC-MS/MS data were processed using ProteinLynx software (Waters-Micromass) and searched against the NCBInr, MSDB, or Swiss-Prot/TrEMBL protein databases using Mascot Search (Matrix Science Ltd., London, UK). The biological function(s) of each protein identified were determined from the Wellcome Trust Sanger Institute and PUMA2 databases, as reported previously (Mangalappalli-Illathu and Korber, 2006).

4.3.9. Experimental replication and statistical analyses

All experimental data represent the arithmetic mean of at least three independent experiments. Differential protein expression was determined from averaged spot volumes from three experimentally-replicated 2D-PAGE gels. Biofilm thickness and cellular dimension data were analyzed using SAS statistical software (version 9.1.3; SAS Institute Inc., Cary, NC), and the Fisher's Least Significant Difference (LSD) method was used to test for significant ($P < 0.05$) differences.

4.4. Results

4.4.1. Shearability of low-flow and high-flow biofilms

Biofilms grown in low-flow and high-flow conditions reached steady-state by ~120 h. The low-flow and high-flow biofilms grown under these conditions achieved the maximum average thickness at ~120 h; the average thickness measurements were estimated on a daily basis over a 168 h growth period (Figure 3.4.3). Low-flow and high-flow biofilms grown for 120 h were chosen for fractionating into shearable and non-shearable regions using the shear-inducing flow, as they were representative of the steady-state condition. The average thickness of low-flow biofilms before application of shear-inducing flow was 9 ± 5 μm (average thickness ± standard deviation; standard error of the mean (SEM) - 0.4), whereas following shear-inducing flow the thickness marginally reduced to 8 ± 2 μm (SEM - 0.1) (Figure 4.4.1). In contrast, the average thickness of high-flow biofilms (43 ± 24 μm; SEM - 2.1) was significantly reduced to 13 ± 6 μm (SEM - 0.5) by shear-inducing flow. Due to low amount of biomass sheared from the low-flow biofilm system, only high-flow biofilms were used in subsequent

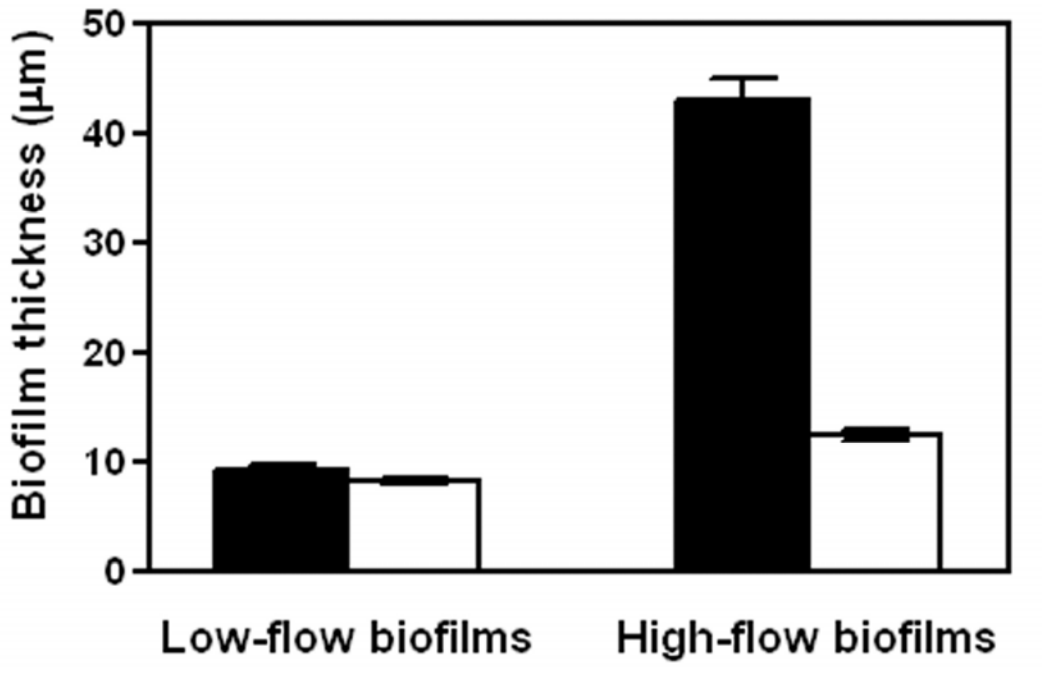


Figure 4.4.1. Mean thickness values (n = 225) of 120 h *Salmonella* serovar Enteritidis low-flow (0.007 cm sec^{-1}) and high-flow (0.07 cm sec^{-1}) biofilms, before (▬) and after (▭) the application of shear-inducing flow (1.33 cm sec^{-1}) for a period of 5 min. Shear-inducing flow separated the high-flow biofilms very efficiently; whereas, the low-flow biofilms were not shearable. The error bars indicate the standard error of the mean.

experimentation. The shear-induced reduction in thickness of high-flow biofilms was significant ($P < 0.05$) (biofilm thickness reduced from 43 ± 2.1 μm (average thickness ± SEM) to 13 ± 0.5 μm); whereas, shear-induced reduction in low-flow biofilm thickness was not significant.

4.4.2. Glycoconjugate composition of shearable and non-shearable regions of high-flow biofilms

Lectin-binding analyses of whole (~0 to 45 μm biofilm depth) and sheared (i.e., non-shearable region; ~0 to 15 μm biofilm depth) high-flow biofilms demonstrated that there was no difference in the EPS glycoconjugate composition of shearable and non-shearable regions (Figures 4.4.2 and 4.4.3; Table 4.4.1). *Glycine max*-CY5, *Triticum vulgaris*-TRITC, and *Ulex europaeus*-FITC showed extensive binding among the eight fluorescently-conjugated lectins analyzed. The whole and sheared biofilms contained 1.2% and 1.4% of GalNAc residues, respectively. The whole biofilms contained 69.1% of fucose residues and 29.7% of GlcNAc2 and NeuNAc residues in combination. The sheared biofilms also contained similar concentrations of the glycoconjugate components (66.5% and 32.1% of fucose residues, and GlcNAc2 and NeuNAc residues in combination, respectively) (Table 4.4.1).

4.4.3. Dimensions of shearable and non-shearable cells of high-flow biofilms

The dimensions and the length:width index (LWI) of biofilm cells (from shearable and non-shearable regions) and planktonic cells (from biofilm effluent and continuous culture) were determined by AFM and image analyses (Figures 4.4.4 and 4.4.5; Table 4.4.2). The average cellular length (l) (± SEM) of biofilm cells from non-shearable and shearable fractions of high-flow biofilms, as well as planktonic cells from biofilm effluent and continuous culture was 2214 ± 231, 2118 ± 53, 2042 ± 53, and 1558 ± 32 nm, respectively. Likewise, the average cellular width (w) (± SEM) of cells from non-shearable and shearable biofilm fractions and planktonic cells from biofilm effluent and continuous culture was determined to be 694 ± 11, 925 ± 10, 928 ± 11, and 914 ± 12 nm, respectively; average cellular depth (d) (± SEM) of cells from non-shearable and shearable biofilm fractions and planktonic cells from biofilm effluent and continuous

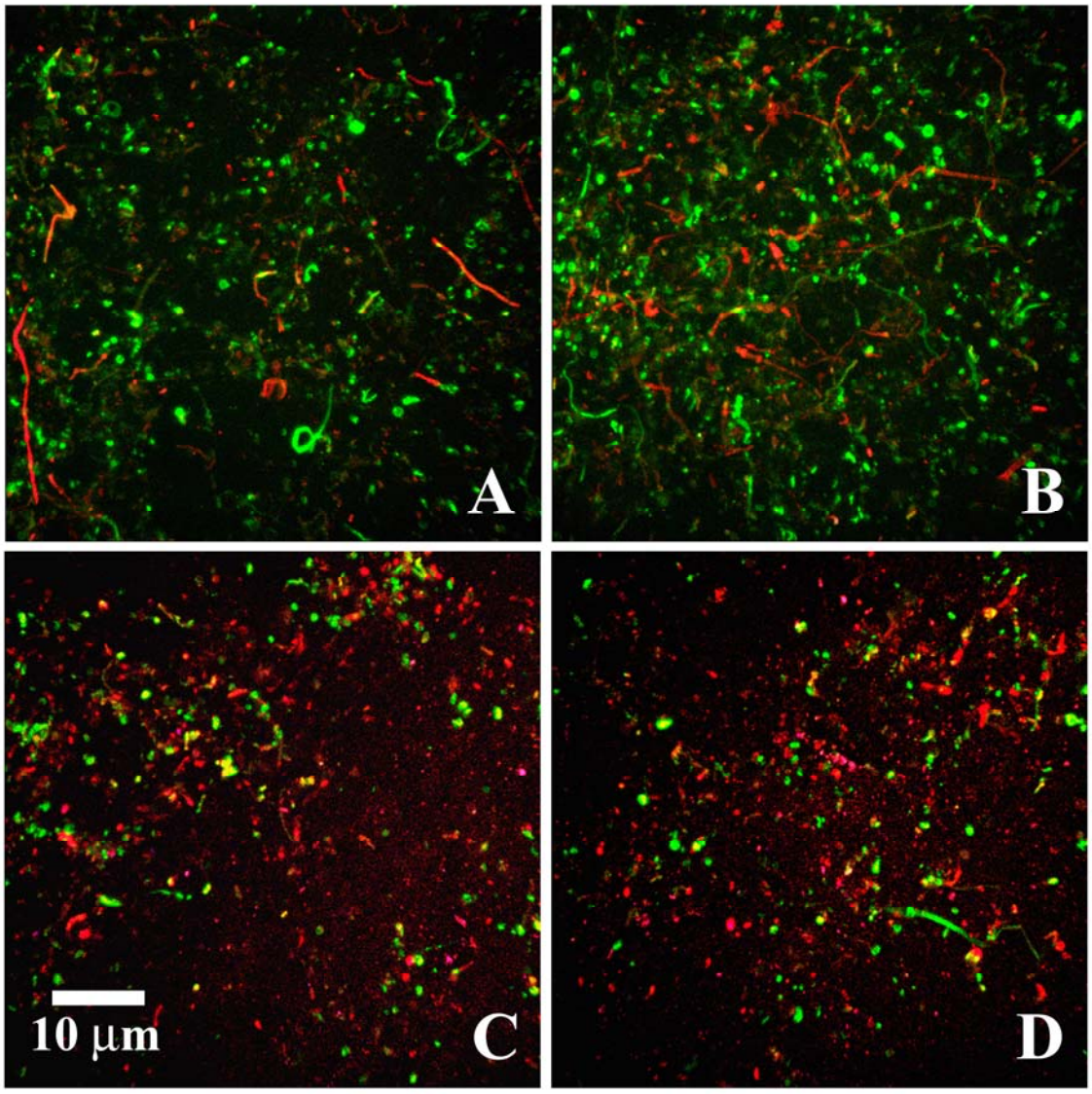


Figure 4.4.2. Representative projected confocal micrographs of lectin-binding analyses, demonstrating the glycoconjugate composition of EPS in 120 h whole (~0 to 45 μm biofilm depth) (A and B) and sheared (~0 to 15 μm biofilm depth) (C and D) *Salmonella* serovar Enteritidis high-flow biofilms. The GalNAc residues of EPS were bound by *Glycine max*-CY5 conjugate (blue), *α*-L-fucose residues were bound by *Ulex europaeus*-FITC conjugate (green), and GlcNAc2 and NeuNAc residues were bound by *Triticum vulgaris*-TRITC conjugate (red). The italicized names indicate the lectin from the source plant, followed by the acronym indicating the labeled fluorescent dye.

Table 4.4.1. Glycoconjugate composition of EPS in 120 h whole high-flow biofilms and non-shearable region of high-flow biofilms of *Salmonella* serovar Enteritidis, as determined by lectin-binding analyses

Glycoconjugates*	Proportion in high-flow biofilms (%)	
	Whole biofilms	Non-shearable region of biofilms
N-acetyl galactosamine (GalNAc)	1.2	1.4
α-L-fucose	69.1	66.5
N-acetyl glucosamine (GlcNAc2) + *N*-acetyl neuraminic acid (NeuNAc)	29.7	32.1

* GalNAc residues are specifically bound by *Glycine max*-CY5 conjugate, *α*-L-fucose residues are specifically bound by *Ulex europaeus*-FITC conjugate, and GlcNAc2 + NeuNAc residues are specifically bound by *Triticum vulgaris*-TRITC conjugate. Italicized name indicates the lectin from the source plant, and the following acronym indicates the labeled fluorescent dye.

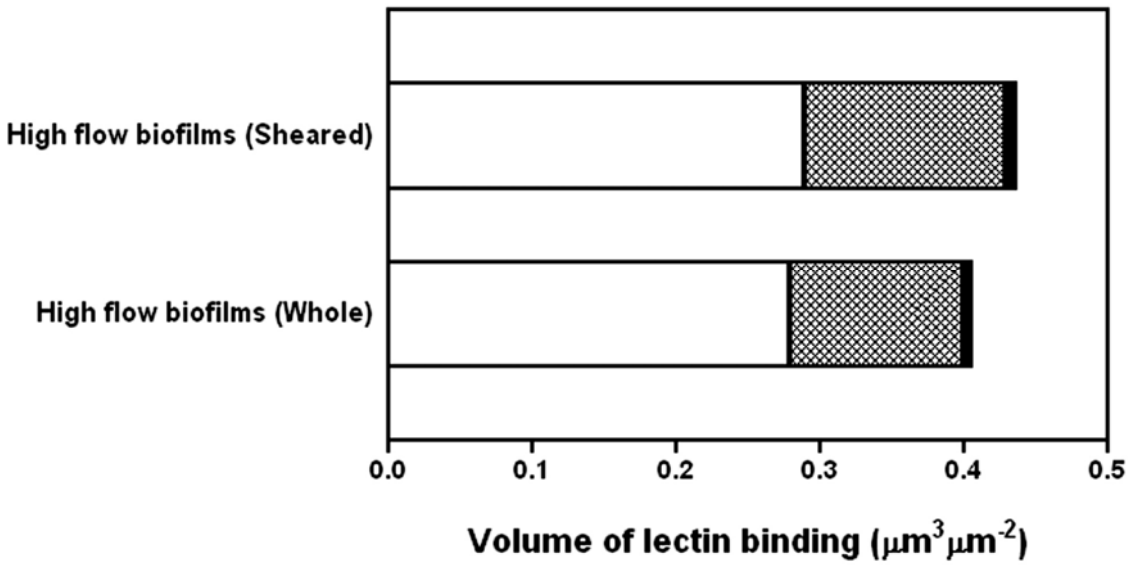


Figure 4.4.3. Proportional distribution of different glycoconjugates in the EPS of 120 h whole and sheared *Salmonella* serovar Enteritidis high-flow biofilms. The symbols (▭), (▨), and (■) indicate the proportion of *α*-L-fucose, *N*-acetyl glucosamine (GlcNAc2) and *N*-acetyl neuraminic acid (NeuNAc) combination, and *N*-acetyl galactosamine (GalNAc), respectively.

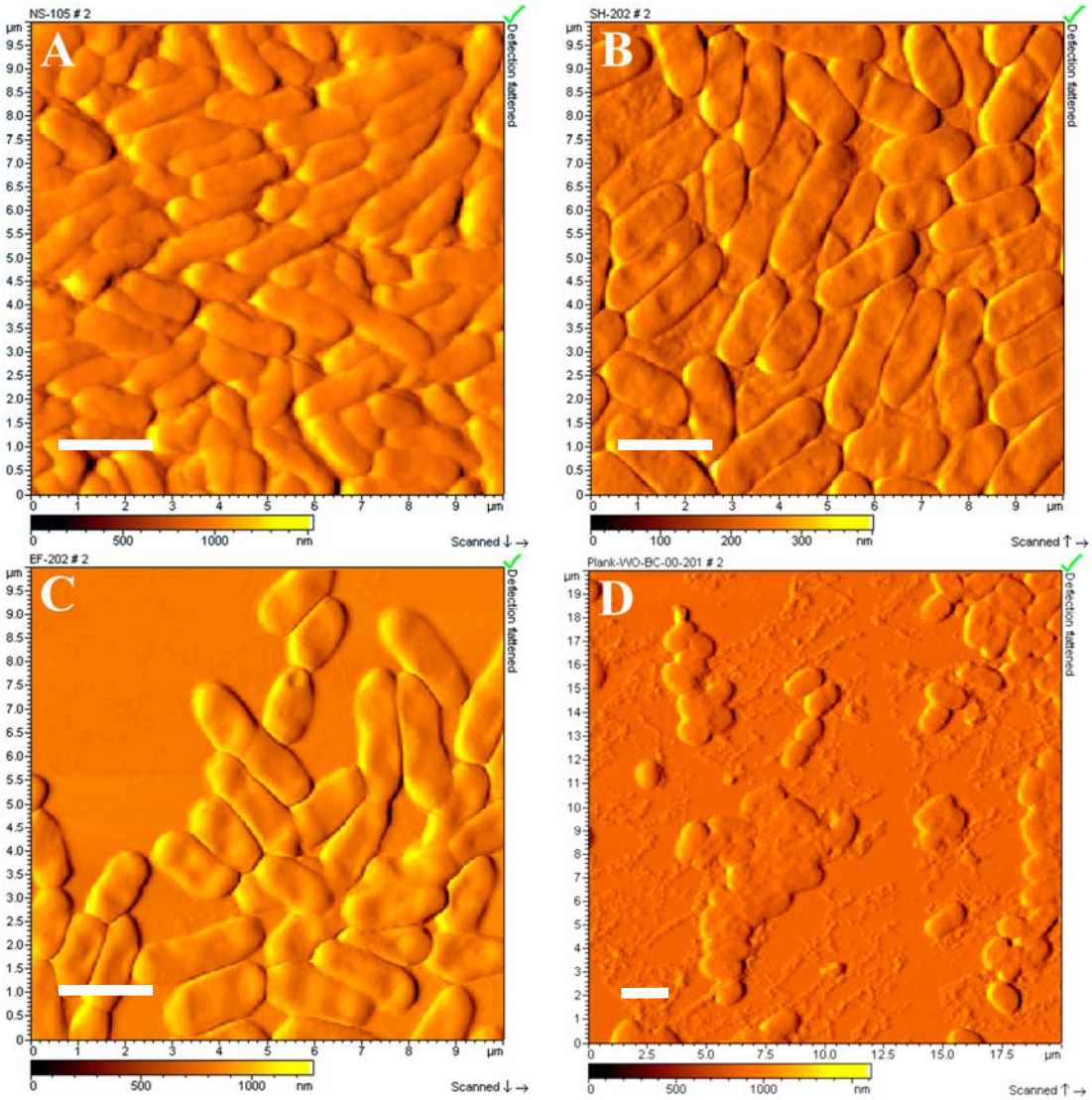


Figure 4.4.4. Atomic force micrographs (AFM; 10 X 10 and 20 X 20 μm^2) rendered in deflection mode of cells from non-shearable region (A), shearable region (B), and effluent (C) of 120 h high-flow *Salmonella* serovar Enteritidis biofilms as well as planktonic cells grown in continuous culture for 120 h (D). The mean cellular dimensions (length and width) of cells from the four sources and the corresponding LWI values are presented in Table 4.4.2. The cells in non-shearable region were variable in length, as indicated by high standard deviation values, with lesser width in comparison to cells from shearable region as well as biofilm effluent. The cells from shearable region of biofilms were apparently similar in appearance to cells in the effluent; LWI values of the two sets of cells also indicated the similarity in their cellular dimensions. Bars in the individual images indicate 2 µm.

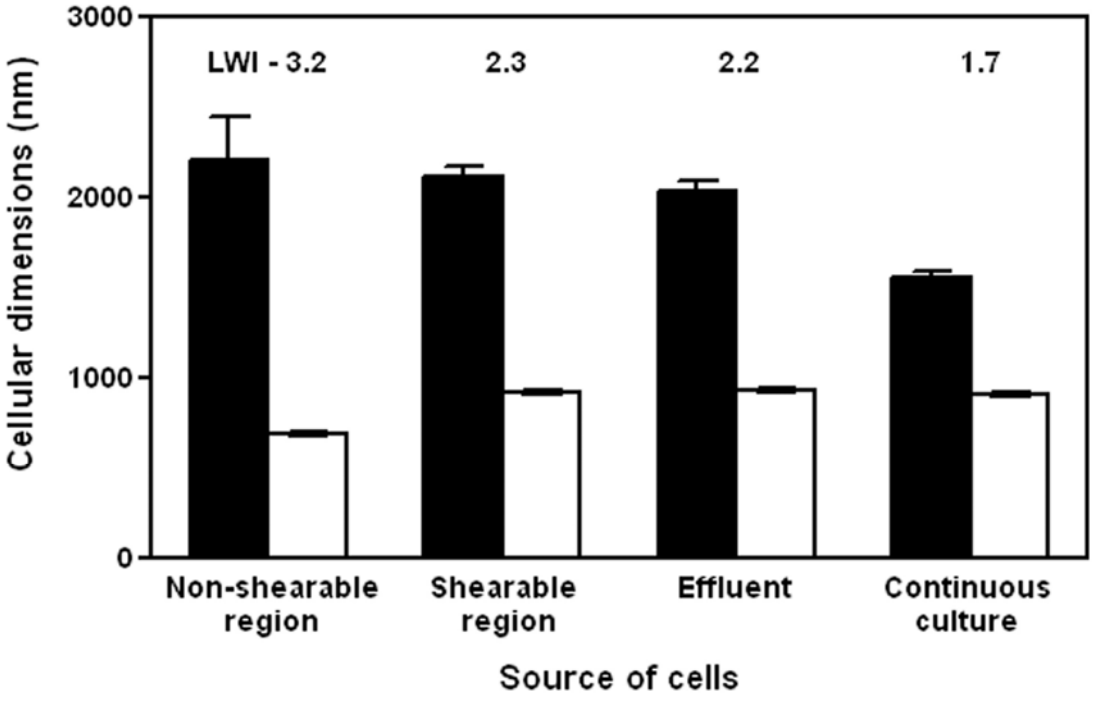


Figure 4.4.5. Mean cellular dimensions (length [▬] and width [▭]) and length:width indices (LWI) of *Salmonella* serovar Enteritidis cells (n = 80) from non-shearable region, shearable region, and effluent of 120 h high-flow biofilms as well as planktonic cells grown in continuous culture for 120 h. The mean cellular dimensions and the corresponding LWI values of cells from all the four sources are illustrated in Table 4.4.2. The error bars indicate the standard error of the mean.

Table 4.4.2. Cellular dimensions of *Salmonella* serovar Enteritidis cells from non-shearable region, shearable region, and effluent of biofilms, as well as planktonic cells grown in continuous culture grown for 120 h

Source of cells	*l** (nm)	*w** (nm)	*d** (nm)	LWI[#]	Average SA (nm^2)	Average V (nm^3)	SA/V (nm^{-1})
Biofilm cells							
Non-shearable region	2214 ± 2062	694 ± 94	333 ± 69	3.2	5.0×10^6	5.1×10^8	9.8×10^{-3}
Shearable region	2118 ± 471	925 ± 86	207 ± 40	2.3	5.2×10^6	4.1×10^8	1.3×10^{-2}
Planktonic cells							
Biofilm effluent	2042 ± 473	928 ± 96	280 ± 37	2.2	5.5×10^6	5.3×10^8	1.0×10^{-2}
Continuous culture	1558 ± 287	914 ± 110	220 ± 39	1.7	3.9×10^6	3.1×10^8	1.3×10^{-2}

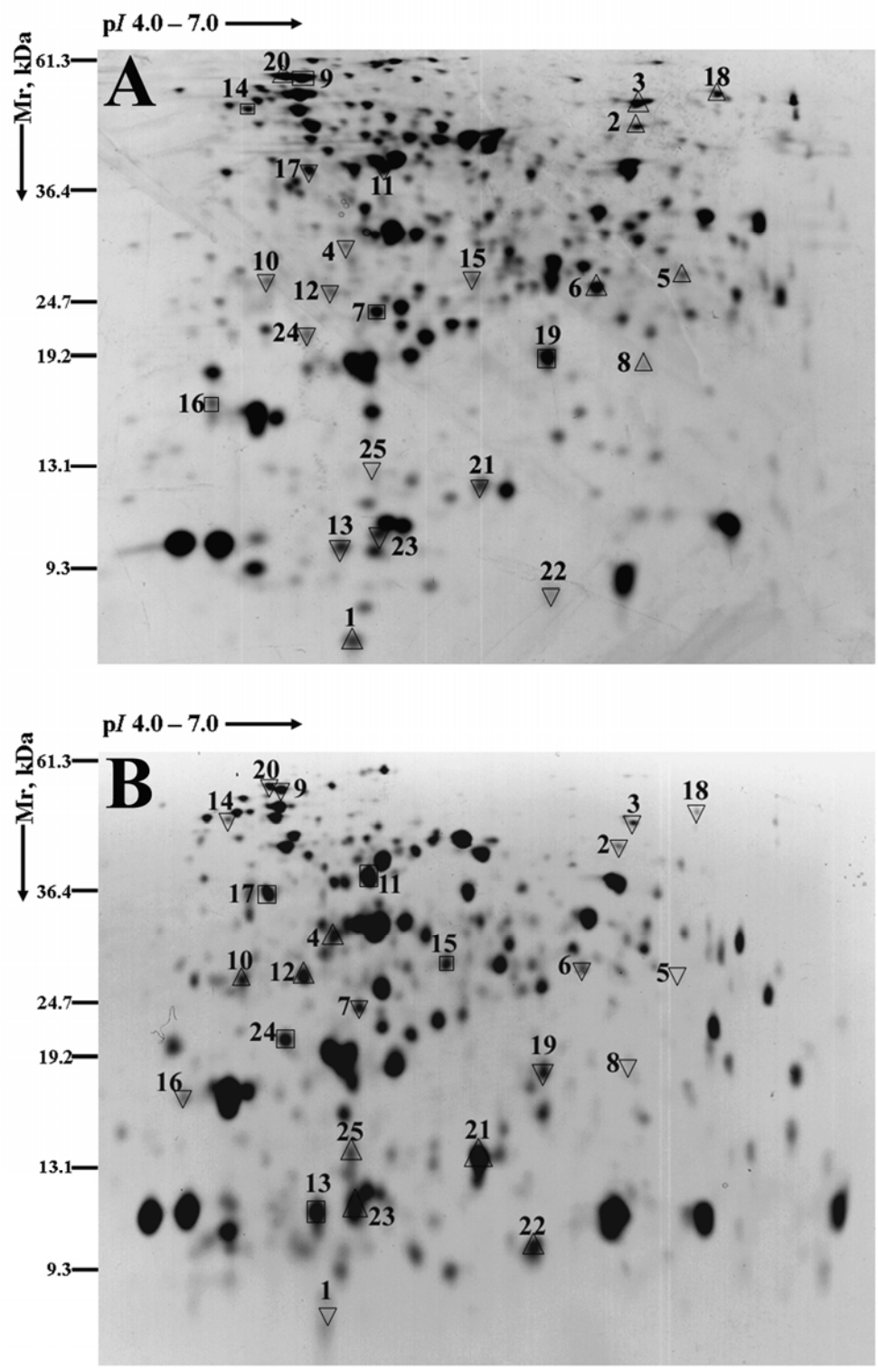
* Arithmetic mean (n = 80) ± standard deviation.
[#] Calculated by dividing mean cell length with corresponding mean cell width.

culture was determined to be 333 ± 15, 207 ± 9, 280 ± 8, and 220 ± 8 nm, respectively. Corresponding standard deviation values pertaining to *l*, *w*, and *d* for each cell sub-population are provided in Table 4.4.2. The average values with respect to cell surface area (SA), cellular volume (V), and SA/V for all four cell sub-populations are also presented in Table 4.4.2.

4.4.4. Protein expression patterns of shearable and non-shearable cells of high-flow biofilms

The protein expression of cells from shearable and non-shearable fractions of high-flow biofilms was compared with a virtual gel averaged from six 2D-PAGE gels; three gels each representing proteins from shearable and non-shearable biofilm fractions (Figure 4.4.6; Table 4.4.3). There were 156 and 191 protein spots detected in gels of shearable region and non-shearable regions, respectively, of which 67 spots were differentially expressed. Using LC-MS/MS, 25 differentially-expressed proteins were identified in the two biofilm regions. Out of the 25 differentially-expressed proteins, seven and eight proteins were relatively increased, 13 and 12 proteins were relatively decreased, and five proteins each were unchanged in their level of expression in shearable and non-shearable biofilm fractions, respectively (Table 4.4.3). Seven proteins increased (CspA, Eno, GrcA, Hns, Tsf, Tuf, and YjgF) in the shearable fraction were found to decrease in the non-shearable fraction; whereas, eight increased proteins (AtpA, DegQ, DnaK, GpmA, OppA, PduA, TpiA, and WrbA) in the non-shearable fraction were found to decrease in the shearable fraction. The proteins that had a relative increase in expression in the shearable region were those involved in adaptation and atypical conditions (CspA and GrcA), broad regulatory functions (Hns), energy metabolism (Eno), protein translation and modification (Tsf and Tuf), and a hypothetical protein (YjgF); whereas, the proteins with increased expression in the non-shearable region were those involved in amino acid biosynthesis (WrbA), degradation of proteins and carbon compounds (DegQ and PduA), energy metabolism (AtpA, GpmA, and TpiA), nutrient (oligopeptide)-binding (OppA), and the chaperone protein Hsp70 (DnaK).

Figure 4.4.6. (facing page) Total proteins of p*I* values ranging from 4 and 7, which were differentially-expressed in non-shearable and shearable regions of *Salmonella* serovar Enteritidis high-flow biofilms. The images illustrate the representative 2D-PAGE gels pertinent to proteins extracted from non-shearable region (A) and shearable region (B) of biofilms. Virtual averaged gel created out of six 2D-PAGE gels replicated experimentally, three gels each representing proteins from non-shearable and shearable regions using Phoretix^{TM} 2D analysis software, was used as the control. The symbol (□) indicates unchanged level of expression, (△) indicates enhanced expression, and (▽) indicates reduced expression on comparison to their expression in the control. The description and fold change in expression of proteins are presented in Table 4.4.3.


pI 4.0 – 7.0
Mr, kDa
61.3
36.4
24.7
19.2
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A
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Table 4.4.3. Proteins that differentially-expressed in cells of non-shearable and shearable regions of *Salmonella* serovar Enteritidis high-flow biofilms grown for 120 h

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	pI[a]	Fold change[b]	
						Non-shearable region	Shearable region
Degradation (carbon compounds)	1	*pduA*	Putative propanediol utilization protein A	9.59	6.72	1.83	0.17
Degradation (proteins, peptides, and glycoproteins)	2	*degQ*	Serine endoprotease	47.28	6.80	1.69	0.31
Energy metabolism (ATP synthesis)	3	*atpA*	ATP synthase α-subunit	54.98	5.80	1.78	0.22
Energy metabolism (glycolysis)	4	*eno*	Enolase	45.47	5.25	0.23	1.77
	5	*gpmA*	Phosphoglycerate mutase	28.36	5.78	1.72	0.28
	6	*tpiA*	Triose phosphate isomerase	26.92	5.68	1.60	0.40
Energy metabolism (non-oxidative phase of pentose phosphate pathway)	7	*rpiA*	Ribose-5-phosphate isomerase A	22.90	5.08	1.36*	0.64
Aromatic amino acid biosynthesis	8	*wrbA*	Flavoprotein WrbA	20.74	5.79	1.58	0.42
Ribosomal protein synthesis and modification	9	*rpsA*	30S ribosomal subunit protein S1	61.25	4.89	1.49*	0.51
Protein translation and modification	10	*tuf*	Elongation factor Tu (fragment)	29.29	5.30	0.37	1.63
	11	*tufA*	Translation elongation factor EF-Tu.A	43.40	5.30	0.64	1.35*
	12	*tsf*	Translation elongation factor Ts	30.36	5.13	0.43	1.57
RNA synthesis, RNA modification, and DNA transcription	13	*rpoZ*	DNA-directed RNA polymerase ω-chain	10.24	4.87	0.52	1.48*

Continued…

Table 4.4.3. continued.

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold change[b]	
						Non-shearable region	Shearable region
Cell envelope (surface structures)	14	*fljB*	Phase 2 flagellin	52.41	4.75	1.45*	0.55
Cell processes (amino acid-binding proteins)	15	*argT*	Lysine-arginine-ornithine-binding periplasmic protein	28.20	5.99	0.56	1.44*
Cell processes (carbohydrate-binding proteins)	16	*crr*	Phosphotransferase system enzyme II (glucose-permease IIA component)	18.12	4.73	1.45*	0.55
	17	*fruB*	Phosphotransferase system enzyme II (fructose-specific IIA/FPr component)	39.59	4.87	0.52	1.48*
Cell processes (binding proteins [other])	18	*oppA*	Oligopeptide-binding protein complexed with Kvk, chain A	58.81	5.85	1.82	0.18
Cell processes (detoxification)	19	*sodB*	Superoxide dismutase [Fe]	21.18	5.58	1.45*	0.55
Cell processes (chaperones)	20	*dnaK*	Chaperone protein Hsp70	69.13	4.83	1.61	0.39
Broad regulatory functions	21	*hns*	DNA-binding protein H-NS (histone-like protein II)	15.41	5.32	0.43	1.57
Atypical conditions and adaptation	22	*cspA*	7.4-kDa cold-shock protein	7.27	5.57	0.16	1.84
	23	*grcA*	Autonomous glycyl radical cofactor	14.34	5.10	0.36	1.64
Hypothetical proteins	24	*ycbL*	Hypothetical protein YcbL (putative metallo-*β*-lactamase)	23.73	4.95	0.51	1.49*
	25	*yjgF*	Conserved hypothetical protein YjgF	13.57	5.36	0.26	1.74

Mr, molecular mass; p*I*, isoelectric point.

[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.

[b] Fold change in protein expression from that of control, as described in materials and methods section.

* Unchanged level of expression (i.e., 0.67 – 1.49).

4.5. Discussion

The effect of system hydrodynamics and behavioral factors on the detachment of biofilm bacteria have previously been examined (Korber et al., 1989; Lawrence et al., 1992; Rupp et al., 2005); however, the effect of fluid velocity on biofilm shearability is only poorly understood. The term "shearability" in the context of the present study indicates the extent of mechanical separation of the biofilms into shearable and non-shearable regions based on changes in hydrodynamic conditions. *Salmonella* serovar Enteritidis biofilms were grown under low-flow and high-flow conditions until they reached a steady-state condition at ~120 h of growth, which was determined based on the pattern of cell shedding (Figure 3.4.1). The average thickness (± standard deviation) of low-flow and high-flow biofilms was determined to be 9 ± 5 μm and 43 ± 24 μm, respectively at this stage of growth (Figure 4.4.1). Thus, shear-inducing flow was applied after 120 h of growth. This was an empirically-determined flow rate of 1.33 cm sec^{-1} applied for 5 min, which was determined based on the ability of flow to separate the low-flow and high-flow biofilms into shearable and non-shearable fractions (Figure 4.2.1). The fractionated material was then used to compare the physiology of cells from shearable and non-shearable biofilm zones with respect to morphological differences and differential protein expression.

The extensive (70%) removal of loosely-attached (shearable) biofilm material from high-flow biofilms followed the application of shear-inducing flow. In contrast, shearing of low-flow biofilms was minimal (11%). The non-shearable region made up only 30% of the total thickness in high-flow biofilms as compared to 89% of the total biofilm thickness in low-flow biofilms. The shear-induced reduction in thickness of high-flow biofilms was significant ($P < 0.05$); whereas, shear-induced reduction in low-flow biofilm thickness was not significant. In addition, the location-to-location variability in biofilm thickness was significantly higher in low-flow and high-flow biofilms before the application of shear-inducing flow (deduced from the high standard deviation values of biofilm thickness), a feature that tended to result in less-variable (in terms of thickness measurements), flatter biofilms following shearing. Stoodley et al. (2002) reported that biofilms grown under turbulent flow conditions were generally more resistant to shearing than biofilms grown under low laminar flow conditions.

However, under the highly laminar flow regimens tested during this study (Reynolds numbers (*Re*) for low-flow and high-flow conditions were 0.237 and 2.37, respectively) the biofilms formed under high-flow were more subject to cell loss due to shearing. This is likely a function of the fact that the high-flow biofilms were significantly more thick, therefore more potentially-shearable biofilm material was present. The average thickness of high-flow biofilms after the application of shear-inducing flow was significantly higher ($P < 0.05$) than the average thickness of low-flow biofilms before shearing clearly demonstrating the growth-stimulating effect of a 10-fold difference in nutrient laminar flow velocity. This also suggested that high-flow biofilms were more firmly adhered to the substratum (possible due to a difference in EPS composition), therefore required more forceful mechanical process for complete removal. This agrees with Donlan and Costerton (2002) comment that biofilms formed in high-shear environments are more resistant to mechanical breakage than those developed under low shear conditions, and has relevance for biofilm control in the food industry.

The formation of elongated structures like filamentous "streamers" which oscillate in the flow, and altered EPS composition providing enhanced viscoelasticity and thereby preventing the shearing and detachment of biofilms while growing in turbulent flow conditions, have been reported (Stoodley et al., 1998, 2002; Donlan and Costerton, 2002). There are also reports that biofilms regulate the EPS strength by increasing EPS synthesis or by regulating metabolic pathways in response to the flow velocity (Applegate and Bryers, 1991; Liu and Tay, 2000; Stoodley et al., 2002). There was a significant difference in the EPS glycoconjugate composition of low-flow and high-flow *Salmonella* serovar Enteritidis biofilms (Figures 3.4.6 and 3.4.7; Table 3.4.1). Thus, the difference in shearability of low-flow and high-flow biofilms might be due to differences in their respective glycoconjugate make-up, in particular, the presence of much higher concentrations of GalNAc residues in the low-flow biofilms. Such an alteration in EPS glycoconjugate composition may represent an adaptive response to the particular flow conditions. However, in the current study, there was no significant difference in the EPS glycoconjugate composition of shearable and non-shearable regions of high-flow biofilms (Figures 4.4.2 and 4.4.3; Table 4.4.1).

In this study, cellular morphology of biofilm cells from shearable and non-shearable regions of high-flow biofilms was investigated using AFM. Bacteria have been shown to alter their cellular dimensions and overall cellular morphology in order to compensate for the deleterious effect of stressors prevailing in the microenvironment; *P. aeruginosa* biofilm cells morphologically adapted to starvation by elongating, which is distinct from the typical starvation response in planktonic bacteria of reducing cell size (Steinberger et al., 2002). It was found that the cells in non-shearable region were longer, but more slender than cells in shearable regions and the cells from the shearable biofilm region were morphologically similar to planktonic cells in the biofilm effluent. However, these cells were morphologically distinct from cells in the non-shearable biofilm region and planktonic cells grown in continuous culture. Cell length was the greatest in cells from the deeper (non-shearable) regions of biofilms, becoming reduced in cells from shearable biofilm regions and even more reduced in the planktonic cells. Notably, cellular width correspondingly increased in the same cells, with the lowest cellular width measurements being found in non-shearable regions of the biofilms and increasing for the cells from the shearable region, and also for the planktonic cells released into the effluent. Based on the changes in LWI values (Table 4.4.2), it may be inferred that the cellular morphological changes were rather sequential from the biofilm to the planktonic phase and that hydrodynamic shear, nutrient availability, spatial limitations, and various other stressors may be driving factors in cellular morphological changes. The distinct cellular morphologies found in different biofilm regions are suggested to be an adaptive response for surviving stress conditions, as reported previously (Steinberger et al., 2002), and thus linked to cell physiology in these different biofilm regions. Biofilm cells of *P. aeruginosa*, under conditions of carbon-nitrogen imbalance, low carbon availability, and increased nutrient diffusional path length, responded by cellular elongation and thus strategically enlarged their surface area (Steinberger et al., 2002). Recently, Riedel and Lehner (2007) reported that *Enterobacter sakazakii* cells exposed to high osmotic stress conditions formed long filaments as a survival mechanism, a response suggested to be mediated through a functional cell division inhibitory system involving MinD/MinC protein complex. However based on proteomic data, the cellular morphological changes observed in the present study could

not be correlated with the up-regulation of any cell division inhibitory system, as suggested by Riedel and Lehner (2007).

Bester et al. (2005) reported that it was inconclusive as to whether cells released from a pseudomonad biofilm could be assigned to a biofilm, planktonic, or a third phenotype. There is evidence in this study that the morphological features of planktonic cells present in the effluent of *Salmonella* serovar Enteritidis biofilms were similar to the biofilm cells from the shearable region; however, these cells had different morphological features from cells from the non-shearable biofilm region and planktonic cells grown in continuous culture. There are some other recent reports not in keeping with the findings of the current study, that cell growth rate in biofilms is greater than the growth rate of their planktonic cell counterparts, and that the biofilms more closely resemble exponentially-growing planktonic cells than do planktonic cells in the stationary phase (Bester et al., 2005; Mikkelsen et al., 2007). The findings of this study indicated that generalizations concerning growth rate and cell division should not necessarily be made to a "whole" biofilm, since the biofilm may be thick enough to have morphologically and physiologically distinct zones. This very much agrees with the concept of the microbial microenvironment, diffusion limitation and the formation of chemical gradients through the activity and metabolism of biofilm bacteria.

Reports exist of biofilms having distinct zones of physiological heterogeneity, including varying degrees of active protein synthesis (Xu et al., 1998; Tolker-Nielsen and Molin, 2000; Werner et al., 2004). Such spatially variable physiology suggests that biofilm architecture may reflect the need for biofilm bacteria to maintain a set of chemical conditions. A recent report by Rani et al. (2007) has demonstrated that the cells in stratified biofilm zones belonged to four distinct states, such as aerobically growing, fermentatively growing, dormant, and dead, as inferred from the spatial patterns of DNA replication, protein synthesis, and O_2 concentration within staphylococcal biofilms. During proteomic analysis, the use of "whole" high-flow biofilm protein as the control was not employed as it was comprised of only 30% non-shearable portion with the remainder (70%) being shearable. Furthermore, a gradient of chemical conditions that exist in biofilms may also contribute to these effects and thus the physiology of cells and their responses may be different within the cell population. There were 25 proteins that

were differentially expressed in the shearable and non-shearable fractions of the high-flow biofilms (Table 4.4.3), clearly indicating that there were differences in the physiological state of cells in the two biofilm regions. Proteomic profile analyses showed that the expression of various stress response proteins of cells in the two biofilm regions was also different. It was found that *Salmonella* serovar Enteritidis biofilm cells adapted to benzalkonium chloride (BC), an antimicrobial compound, had enhanced expression of CspA, Eno, Tsf, Tuf, and YjgF, and reduced expression of AtpA, DegQ, DnaK, GpmA, and OppA (Mangalappalli-Illathu and Korber, 2006). Thus, there are some similarities in the proteomes of BC-adapted biofilm and the shearable biofilm region of *Salmonella* serovar Enteritidis. Up-regulation of the cold-shock protein, CspC was reported in *E. sakazakii* cells following physical desiccation (Riedel and Lehner, 2007). Thus, cold-shock response and relatively enhanced protein biosynthesis might be features of response of cells in the shearable biofilm region. In contrast, increased proteolysis and degradation of carbon compounds in the non-shearable region could be due to the enhanced expression of DegQ and PduA, respectively. Sampathkumar et al. (2004) reported that *Salmonella* serovar Enteritidis cells exposed to a sub-lethal concentration of trisodium phosphate induced thermotolerance. In this study, the enhanced expression of DnaK in the non-shearable biofilm region suggested that the cells experienced stress associated with heat-stress and/or denaturation along with errors in protein biosynthesis leading to the accumulation of abnormal or misfolded proteins within the cells (Riedel and Lehner, 2007). Enhanced expression of Hns, the "pleiotropic regulator" in the shearable region, is suggestive that stress associated with high-flow may have impacted nuclear DNA; the DNA-binding protein, Hns is involved in maintaining nucleoid organization and regulating DNA repair in bacteria (Riedel and Lehner, 2007). Differential expression of enzymes involved in energy metabolism (AtpA, Eno, GpmA, and TpiA), nutrient-binding (OppA), and amino acid and protein biosynthesis (Tsf, Tuf, and WrbA) in the two biofilm regions further suggest that there were apparent differences in the metabolism of *Salmonella* serovar Enteritidis cells in the biofilm fractions, presumably because the stress conditions within the two regions were not the same. For example, cells in the shearable region might be experiencing

more fluid shear stress, whereas cells in the non-shearable region were experiencing nutrient deprivation and spatial limitation.

In conclusion, there is evidence for the existence of physiologically-distinct regions within *Salmonella* serovar Enteritidis biofilms that reflect biofilm chemical and physical conditions in those regions, something in keeping with changes in cellular morphology. Flow velocity influenced the overall biofilm thickness and exopolymer glycoconjugate composition which, in turn, influenced biofilm shearability. The differences in thickness and shearability of low-flow and high-flow biofilms have significance in the persistence of the biofilms in food processing environments, since the growth conditions (e.g., hydrodynamic shear/disturbances and nutrient status) of the microenvironment could significantly influence the survival and dissemination of biofilms to the surrounding areas. Regions with increased flow could result in thicker, more rigid biofilms that subsequently are more resistant to sanitation measures. The proteomic profile analyses indicated that there was only some minor shift in the metabolic pathways used by biofilm cells from shearable and non-shearable regions; however, the cells from the two regions were physiologically distinct. There are indications that the cells in the two regions have utilized different pathways, especially for stress response, energy metabolism, and degradation of carbon compounds and proteins. The distinct morphology and physiology of cells in the two biofilm regions also indicated that the stress experienced by the cells in the two regions could differ. Various stressors (hydrodynamic shear, spatial limitation, nutrient and O_2 depletion, etc.) in the microenvironment plausibly influenced the morphological and physiological state of cells in the two biofilm zones; consistent with compensatory adaptive mechanisms to overcome the harmful effects of stress.

4.6. Connection to the next study

Biofilms adapt to the nutrient flow velocity by changing biofilm structure as well as altering cellular morphology and physiology. The next study was performed to elucidate how the biofilms respond to sub-lethal and lethal exposure to the antimicrobial compound, benzalkonium chloride.

5. ADAPTIVE RESISTANCE AND DIFFERENTIAL PROTEIN EXPRESSION OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS BIOFILMS EXPOSED TO BENZALKONIUM CHLORIDE

5.1. Abstract

Development of adaptive resistance of *Salmonella enterica* serovar Enteritidis ATCC 4931 biofilms following either continuous (1 μg ml^{-1}) or intermittent (10 μg ml^{-1} for 10 min daily) exposure to benzalkonium chloride (BC) was examined. Biofilms adapted to BC over a 144 h period could survive a normally-lethal BC challenge (500 μg ml^{-1} for 10 min) and then re-grow, as determined by increases in biofilm thickness, total biomass, and ratio of viable to non-viable biomass. Exposure of untreated control biofilms to the lethal BC challenge resulted in biofilm erosion and cell death. Proteins found to be up-regulated following BC adaptation were those involved in energy metabolism (TpiA and Eno), amino acid and protein biosynthesis (WrbA, TrxA, RplL, Tsf, Tuf, DsbA, and RpoZ), nutrient-binding (FruB), adaptation (CspA), detoxification (Tpx, SodB, and a probable peroxidase), and degradation of 1,2-propanediol (PduJ and PduA). A putative universal stress protein (YnaF) was also found to be up-regulated. Proteins involved in proteolysis (DegQ), cell envelope formation (RfbH), adaptation (UspA), heat-shock response (DnaK), and broad regulatory functions (Hns) were found to be down-regulated following adaptation. An overall increase in cellular protein

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This chapter is reproduced with permission from Antimicrob. Agents Chemother. 2006, 50:3588–3596. The publication was co-authored by D. R. Korber. All experimental works with the exception of mass spectrometry analyses were carried out by me. In addition, I wrote the initial draft of the manuscript. Minor modifications were made to the published version to maintain thesis format and style.

biosynthesis was deduced from the significant up-regulation of ribosomal subunit proteins, translation elongation factors, and amino acid biosynthesis protein as well as down-regulation of serine endoprotease. Cold-shock response, stress response, and detoxification are suggested to play roles in the adaptive resistance of *Salmonella* serovar Enteritidis biofilms to BC.

5.2. Introduction

Growing concerns over the transmission of foodborne illnesses have led to a rapid increase in the application of antimicrobial agents in industry and homes. There are concerns that the indiscriminate and inappropriate use (inadequate concentrations, insufficient cleaning before the application, or sub-lethal residual disinfectants remaining following cleaning) of these biocidal compounds may contribute to the spread of bacterial resistance to these compounds, as well as cross-resistance to certain therapeutic antibiotics. A number of studies have demonstrated the potential for this phenomenon to occur (Russell et al., 1998; Russell, 2000; Braoudaki and Hilton, 2004). Cationic agents such as quaternary ammonium compounds (QACs), chlorhexidine, and triclosan have been implicated as the possible causes for the selection and persistence of bacterial strains with antibiotic and biocidal resistance mediated through nonspecific alteration of the cell envelope, degradation, and active efflux (McDonnell and Russell, 1999; Russell, 2002; Braoudaki and Hilton, 2004). Increased tolerance or resistance to oxidizing biocides mediated through production of neutralizing enzymes and DNA repair enzymes has been reported in *Salmonella* (Farr and Kogoma, 1991; Seymor et al., 1996; Mokgatla et al., 1998). Bacterial biofilms have been identified as the most important example of how physiological (phenotypic) adaptation could play a role in conferring intrinsic resistance (Brown and Gilbert, 1993; McDonnell and Russell, 1999). It is further believed that various stresses (chemical stress, desiccation, and starvation) experienced by the surface-associated microorganisms are conducive for the development of enhanced resistance of *Salmonella* to disinfection (Leriche and Carpentier, 1995; Szomolay et al., 2005). However, the exact mechanisms of how such stress conditions lead to enhanced resistance of biofilms to antimicrobial agents remains unclear.

Benzalkonium chloride (BC) is a surface-active QAC commonly used as a cationic surfactant and disinfectant for processing lines and surfaces in the food industry. It also is used as a general clinical disinfectant and antiseptic in healthcare facilities and domestic households, and as an antimicrobial preservative in drugs (Adair et al., 1969; Hoffmann et al., 1973; Simões et al., 2005). Due to their positive charge, QACs form electrostatic bonds with negatively-charged sites on bacterial cell walls, destabilizing the cell wall and cytoplasmic membrane leading to cell lysis, leakage, and death (McDonnell and Russell, 1999; Simões et al., 2005). QACs are bacteriostatic at low concentration and bactericidal at high concentration (Maxcy et al., 1971) and thus low concentrations of this agent may favor the development of adaptive resistance. Adaptive resistance to QACs by prolonged sub-lethal exposure, and the cross-resistance of adapted strains to antimicrobial chemotherapeutic agents (amoxicillin, clavulanic acid, chloramphenicol, imipenem, polymyxin B, tetracycline, and trimethoprim) and other biocides (chlorhexidine and triclosan), has been documented in various bacteria including *S. marcescens* (Chaplin, 1952), *E. coli* (Maxcy et al., 1971; Braoudaki and Hilton, 2004; Langsrud et al., 2004), *P. aeruginosa* (Hoffmann et al., 1973; Loughlin et al., 2002), *P. fluorescens* (Simões et al., 2005), and *S. enterica* (Braoudaki and Hilton, 2004).

It is widely accepted that bacteria living in biofilms are more resistant to chemical, physical, and mechanical stresses than their planktonic counterparts (Li et al., 2001; Szomolay et al., 2005). More recently, it has also been shown that different bacteria undergo transitions from the planktonic to biofilm mode of growth that includes the timed expression of different sets of genes and proteins (Sauer and Camper, 2001; Sauer et al., 2002). A number of proteins have been found to be up-regulated in bacterial cells adapted to QACs (To et al., 2002; Langsrud et al., 2003a; Tabata et al., 2003). Various resistance mechanisms, like changes in membrane fatty acid composition, efflux pumps, degradation of the biocides, slime formation, and modified targets in combination, probably contribute to adaptive resistance to QACs (Langsrud et al., 2003b). Notably, few studies have examined the phenomenon of adaptive resistance of *S. enterica* to BC, as well as to other antimicrobial agents.

In the context of this study, the term "adaptive resistance" refers to physiological or phenotypic resistance that developed in an organism following prolonged exposure to an antimicrobial agent. This study aimed at understanding the mechanisms of development of adaptive resistance or tolerance in *Salmonella* serovar Enteritidis biofilms after prolonged sub-lethal exposure (either continuously or intermittently) to BC, based on their ability to survive subsequent lethal treatment and to re-grow in the presence of BC. Comparative analysis of the protein expression patterns of BC-adapted and untreated biofilm cells was performed to elucidate significant biochemical patterns during the development of adaptive resistance to BC.

5.3. Materials and methods

5.3.1. Media and chemicals

Tryptic Soy Agar (TSA), Standard Plate Count Agar (SPCA), and Trypticase Soy Broth (TSB) were purchased from BBL (Becton Dickinson, Cockeysville, MD); benzalkonium chloride (BC; $C_6H_5CH_2N(CH_3)_2RCl$ (R = C_8H_{17} to $C_{18}H_{37}$)), magnesium chloride ($MgCl_2$), phenylmethylsulphonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), DNase, RNase A, fluorescein sodium salt, bromophenol blue, DL-dithiothreitol (DTT), and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO); sodium chloride was from EM Science (Gibbstown, NJ); EDTA was from J. T. Baker Chemical Co. (Philipsburg, NJ); glycerol, sodium dodecyl sulphate (SDS), Tris base, and urea were purchased from Life Technologies (Grand Island, NY); *Bac*Light™ Live/Dead Viability Probe was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada); and immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0), Immobiline DryStrip gels, and PlusOne™ Protein Silver Staining Kit were purchased from GE Healthcare Bio-Sciences Inc. (Baie d'Urfé, QC, Canada).

5.3.2. Bacteria and culture conditions

Salmonella enterica serovar Enteritidis ATCC 4931 (hereafter referred to as *Salmonella* serovar Enteritidis) was cultured from a frozen stock on TSA plates

overnight at 37^{o}C. Cells in the mid-log phase of growth were obtained by transferring a loopful of colony material from TSA plates to 50 ml of 10% [wt/vol] TSB in an Erlenmeyer flask and incubating on a gyratory shaker (150 ± 5 rpm) held at room temperature (RT; 21 ± 2^{o}C) for approximately 12 h. These cells, which were determined to be in the mid-log phase of growth via growth curve experiment, were used to inoculate flow cells.

5.3.3. Flow cells, inoculation, and flow velocity

Multi-channel flow cells were constructed using polycarbonate sheets into which channels were milled, as described previously (Korber et al., 1994). Flow cell channels were sterilized by flushing with 5.25% [wt/vol] sodium hypochlorite solution for 10 min. Reservoirs of sterile nutrient medium (10% TSB) were connected via silicone tubing to the flow cell channels and subsequently connected to the waste reservoir. Medium was pumped at a rate of 0.07 cm sec^{-1} through flow cells using a Watson-Marlow peristaltic pump (Model 202U; Watson-Marlow, Cornwall, UK). Each flow cell channel was separately inoculated with 0.5 ml mid-log phase *Salmonella* serovar Enteritidis cells, prepared as outlined above, concentrated or diluted to an optical density equivalent to 0.5 McFarland standard (1.5 X 10^{8} cfu ml^{-1}).

5.3.4. Determination of minimum inhibitory concentration (MIC) and lethal concentration of BC

The MIC of BC for planktonic cells of *Salmonella* serovar Enteritidis was determined to be 15 µg ml^{-1} by doubling dilution-based nephalometry (Figure 5.3.1), coupled with plating of mid-log phase planktonic cells treated with increasing concentrations of BC on SPCA. Based on preliminary MIC experiments using biofilms, sub-lethal concentrations of 1 and 10 µg ml^{-1} were selected for continuous and intermittent BC applications, respectively. The untreated control biofilms were unable to withstand continuous exposure to 5 and 10 µg ml^{-1} BC. The concentration of BC for lethal challenge was determined as 500 µg ml^{-1} BC, based on the preliminary observations and recommendations of regulatory agencies for surface disinfection using QACs (Health Canada, 1999; Food and Agriculture Organization, 2003).

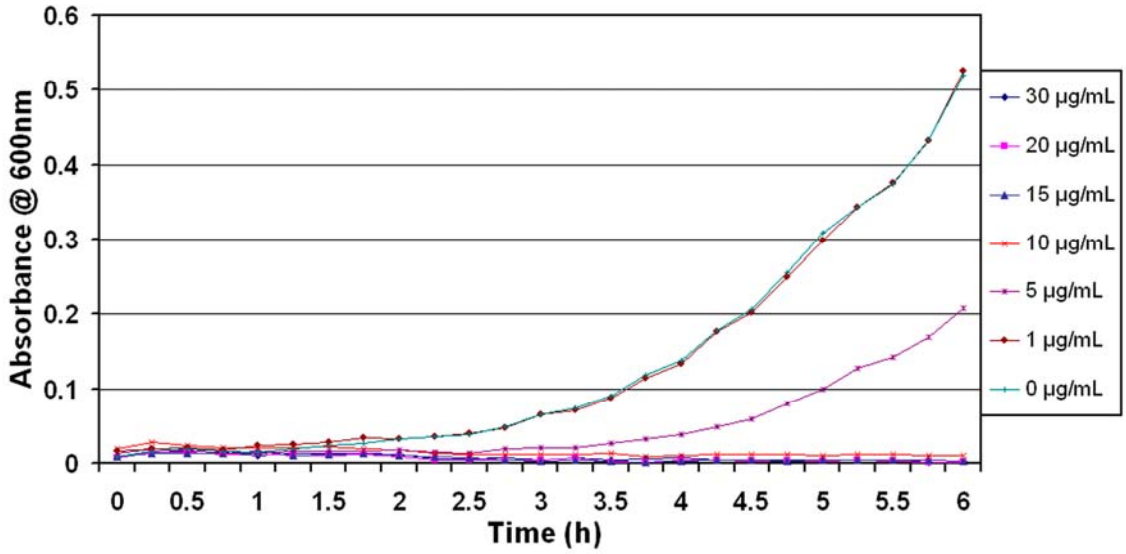


Figure 5.3.1. Determination of MIC of benzalkonium chloride (BC) on the stationary phase planktonic cells of *Salmonella* serovar Enteritidis, as determined by nephalometry.

5.3.5. Adaptation of *Salmonella* serovar Enteritidis biofilms to BC

Established (24 h) biofilms grown under laminar flow conditions were exposed to sub-lethal concentrations of BC either continuously (1 μg ml^{-1}) or intermittently (10 μg ml^{-1} for 10 min daily) over an additional 144 h period. After 168 h, the biofilms were challenged with a lethal concentration of BC (500 μg ml^{-1} for 10 min) and allowed to re-grow for 24 h in a BC-free flowing environment. The biofilms were then continuously exposed to 5 μg ml^{-1} BC for another 24 h period. The ability of the biofilms to survive exposure to 500 μg ml^{-1} BC and to re-grow first in BC-free, and then in a BC-containing, environment was determined using biofilm thickness measurements and confocal laser scanning microscopy (CLSM; *see* below) analyses. Biofilms grown for proteomic analysis were adapted to BC, either continuously or intermittently for 144 h, after which biofilms were disrupted and protein extracted. Experiments using untreated control biofilms were performed throughout the study. A schematic illustration of BC adaptation and lethal challenge experiments on *Salmonella* serovar Enteritidis biofilms is provided in Figure 5.3.2.

5.3.6. Dark-field microscopy

A Zeiss model III RS microscope equipped with a 10X dark-field (0.22 numerical aperture) objective lens and a cooled CCD camera (Sensys model 1401E; Photometrics, Tucson, AZ) controlled by PC-based RS Image version 1.7.3 software (Roper Scientific Inc., Trenton, NJ) was used to collect images for low-power montage reconstruction. Montages of approximately 1 X 10^6 μm^2 in total area were assembled using Adobe Photoshop version 7.0 software (Adobe Systems Inc., Mountain View, CA).

5.3.7. Biofilm thickness measurements

The thickness of biofilms was measured in micrometers using a computer controlled, motorized *z*-axis stepper motor and manual focusing using a Nikon microphot-FXA microscope (Nikon Corp., Tokyo, Japan) (Korber et al., 1994). Fifteen random fields were assessed for each biofilm with five separate thickness values

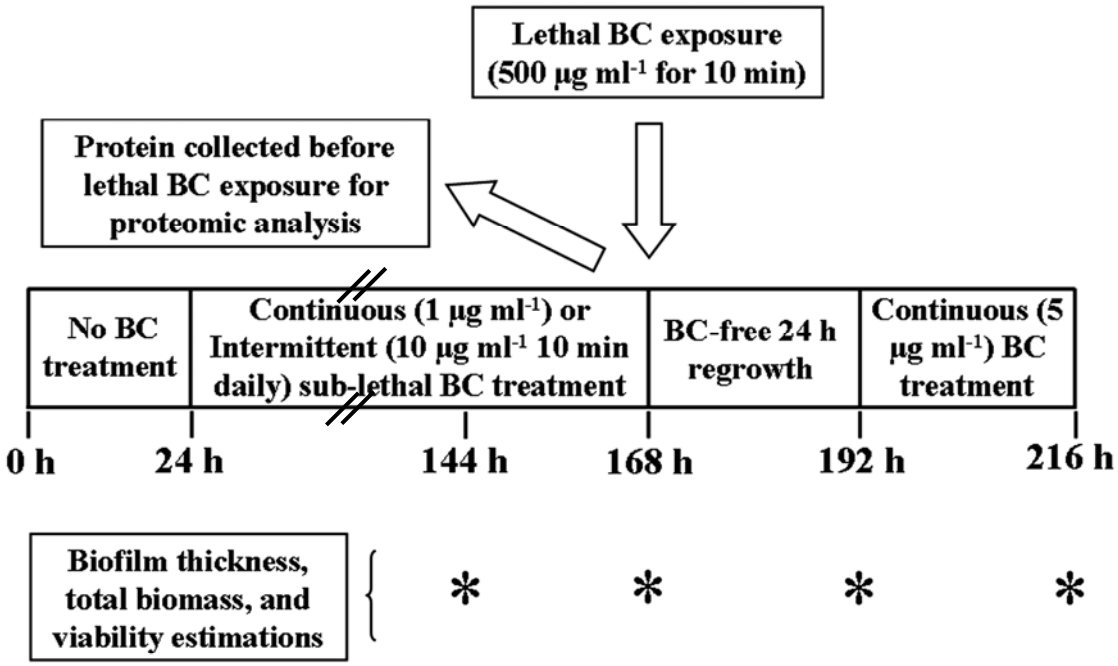


Figure 5.3.2. Schematic illustration of BC adaptation and lethal challenge experiments on *Salmonella* serovar Enteritidis biofilms grown for 24 h in TSB.

obtained per field (n = 75). These values were averaged to obtain the thickness of each biofilm.

5.3.8. CLSM, fluorescent probes, and digital image analyses

Optical thin sections (OTSs) were acquired for estimating biofilm biomass and viability of cells using a Bio-Rad MRC-600 Lasersharp fluorescence scanning confocal laser system (Korber et al., 1993, 1994). Optical thin sections (each a total of 8067 μm^2) from 5 biofilm optical thin sectioning depths (0, 3.7, 7.4, 11.1, and 14.8 μm, where 0 μm represents the attachment surface) were collected for each of 15 biofilm sampling locations (Korber et al., 1993). Fluorescein was used for negative staining of biofilms and subsequent CLSM for biomass estimation (Caldwell et al., 1992b). In this approach, biomass represents the amount of negatively-stained biofilm material present at each optical thin sectioning depth in terms of area (μm^2) occupied by cell material, and thus is not a volumetric measurement. The ratio of viable to non-viable biomass was also based on the amount of area occupied by either living or dead cell material (μm^2) at each specific optical thin sectioning depth and was thus determined similarly; these biofilms were stained with the *Bac*Light™ Live/Dead Viability Probe and assayed using dual-channel CLSM, and their fluorescence response quantified (Korber et al., 1996, 2002; Webb et al., 2003). Single or dual-channel images were acquired in either the *xy* or vertical *xz* plane of analysis. The analyses of the images for biofilm biomass and viability estimations were performed using MacIntosh™-based NIH image version 1.63f software (National Institutes of Health, Bethesda, MD).

5.3.9. Sample preparation for 2D-PAGE of total cellular proteins

Adapted and untreated control biofilms were aseptically scraped from the flow cell channels. The 2D-PAGE sample preparation was carried out as detailed previously (Sampathkumar et al., 2004). Following protein extraction, the dye-binding assay of Bradford was performed to quantify the protein concentration using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) (Bradford, 1976).

5.3.10. 2D-PAGE and analysis of protein spots

Protein extracts were subjected to high-resolution 2D-PAGE as described by O'Farrell (O'Farrell, 1975) and as modified by Görg et al. (Görg et al., 2000). Isoelectric focusing was performed using Immobiline DryStrips (p*I* 4.0 to 7.0) in conjunction with a Multiphor II electrophoresis unit (GE Healthcare Bio-Sciences Inc.). Equilibrated isoelectric focused strips were placed on 14% [wt/vol] SDS-polyacrylamide gel for second-dimension electrophoresis with Mini-Protean II electrophoresis system (Bio-Rad Laboratories) at a constant 100 V at RT. After electrophoresis, the gels were silver stained in accordance with the manufacturer's instructions, scanned on an Epson 1200C scanner with a transparency adapter as 8-bit grayscale 300 dpi images, and stored. Differentially expressed proteins were then detected and quantified from the stored images with Phoretix^{TM} 2D version 2004 analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). An increase in the protein spot volume of 1.5-fold or more was interpreted as up-regulation whereas a decrease in the spot volume of 1.5-fold or more was interpreted as down-regulation.

5.3.11. Protein identification

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed using a capLC interfaced to a Q-ToF Ultima Global hybrid tandem mass spectrometer fitted with a *Z*-spray nanoelectrospray ion source (Waters-Micromass, Manchester, UK). Protein spots for MS analysis were collected from freshly-prepared 2D-PAGE gels that stained by a modified silver staining protocol provided by GE Healthcare Bio-Sciences Inc. Protein spots of interest were excised from the gel, destained, and in-gel digested with trypsin in accordance with the established protocols for the MassPrep robotic workstation (Waters-Micromass, Manchester, UK). LC-MS/MS data were processed using ProteinLynx software (Waters-Micromass, Manchester, UK) and searched against the NCBInr (http://www.protein.sdu.dk/gpmaw/GPMAW/Databases/NCBInr/ncbinr.html), MSDB (http://csc-fserve.hh.med.ic.ac.uk/msdb.html), or Swiss-Prot/TrEMBL (http://www.expasy.org/sprot) protein databases using Mascot Search (Matrix Science Ltd., London, UK). The biological functions of each protein identified were determined from the Wellcome Trust Sanger

Institute (http://www.sanger.ac.uk) and PUMA2 (http://compbio.mcs.anl.gov/puma2/cgi-bin/index.cgi) databases.

5.3.12. Experimental replication

All BC adaptation and lethal challenge data represent the average of at least three experiments. Differential protein expression of BC-adapted biofilms was determined from averaged spot volumes from four gels replicated experimentally, with a maximum variation of 30% in spot volume between the gels.

5.3.13. Statistical analyses

Biofilm thickness, biomass, and viability data were analyzed using SAS version 9.1.3 statistical software (SAS Institute Inc., Cary, NC), and the Fisher's Least Significant Difference (LSD) method was used to test for significant ($P < 0.05$) differences.

5.4. Results

5.4.1. Effect of BC adaptation and lethal challenge on biofilm thickness

Untreated control biofilms were reduced in thickness and biomass by ~50% following continuous treatment with 10 μg ml^{-1} BC over a 48 h period (Figure 5.4.1). When biofilms were continuously-treated with 1 μg ml^{-1} BC, the average thickness (± standard error) at 168 h was 34 ± 1 μm. Immediately after treatment with a lethal BC challenge (500 μg ml^{-1} for 10 min), the average biofilm thickness was reduced to 25 ± 1 μm (26% reduction) (Figure 5.4.2). Slight re-growth (~5%) was observed over the 24 h period following removal of the BC stress. On subsequent continuous exposure to 5 μg ml^{-1} of BC, the biofilms continued to grow, reaching 37 ± 1 μm at 216 h. When intermittently-treated (10 μg ml^{-1} BC for 10 min daily) biofilms were treated with the lethal BC challenge at 168 h, the average biofilm thickness value was reduced from 17 ± 2 μm to 9 ± 1 μm (47% reduction) (Figure 5.4.2). The proportion of re-growth following lethal BC challenge was found to be greater for the intermittently-treated biofilms (143%

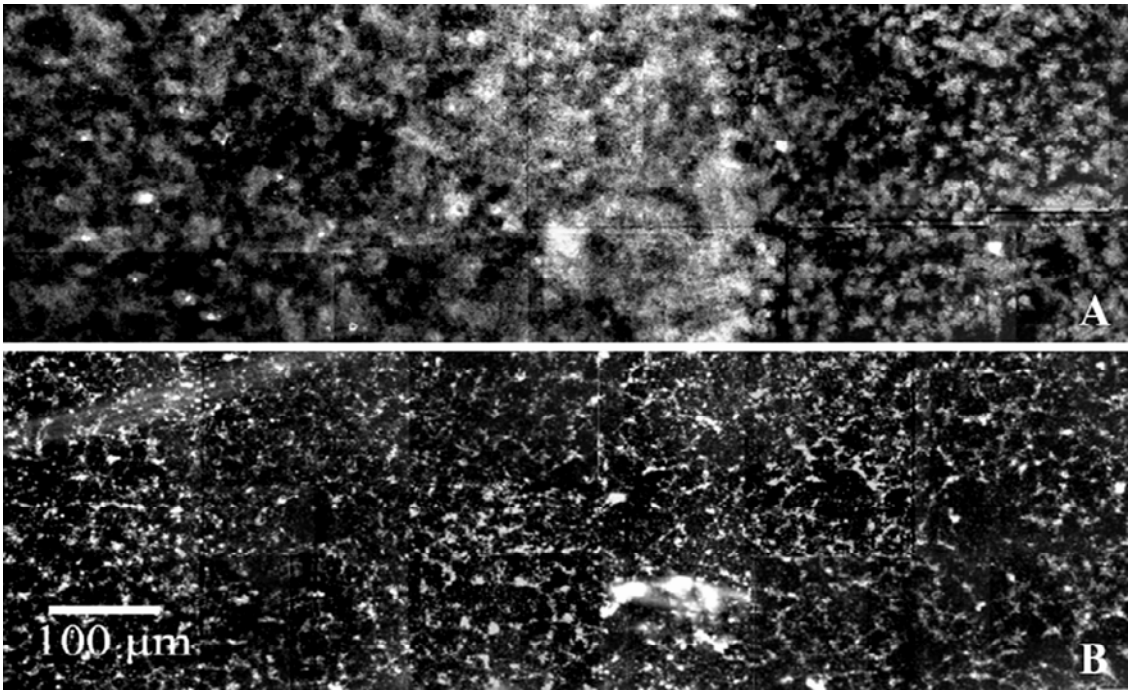


Figure 5.4.1. Low-power dark-field micrograph montages of a biofilm area (A) in an established *Salmonella* serovar Enteritidis biofilm, and (B) the same area of biofilm after 48 h continuous treatment with sub-lethal concentration (10 µg ml^{-1}) of BC. The initially thick biofilm has been reduced to much thinner biofilm due to erosion and loss of biomass.

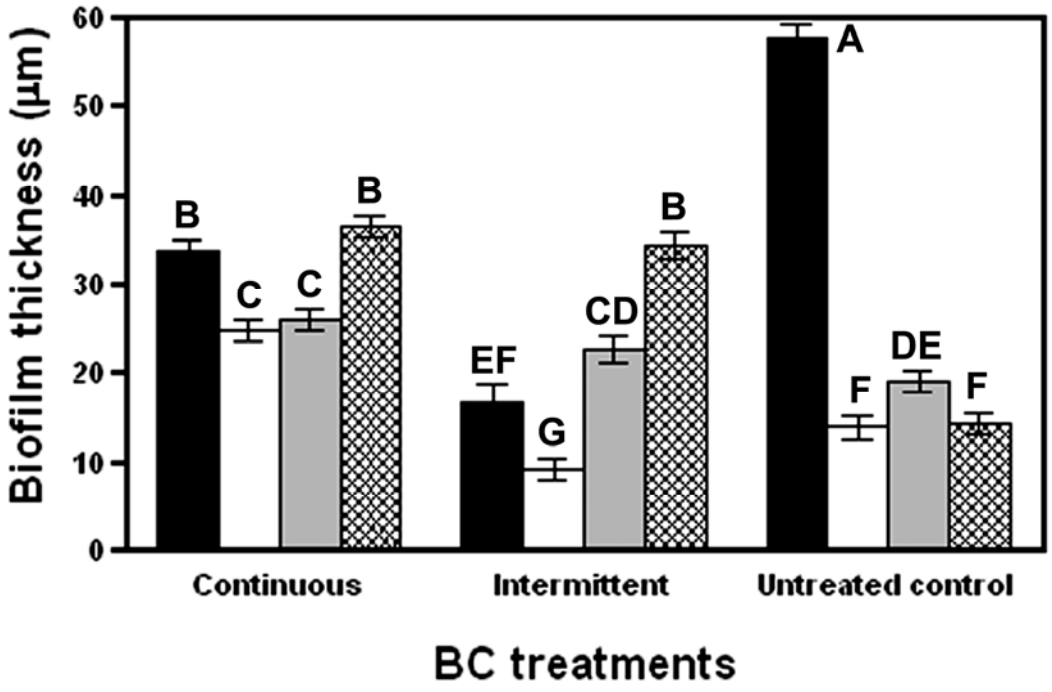


Figure 5.4.2. Thickness of biofilms treated either continuously or intermittently with sub-lethal concentration of BC, or that of the untreated control. The thickness measurements at each time interval are the average of 225 thickness measurements made at random locations from three biofilms replicated experimentally. The symbol (■) indicates average thickness at 168 h before lethal BC treatment (500 μg ml^{-1}), (▭) indicates average thickness immediately after lethal treatment, (▭) indicates average thickness 24 h after lethal treatment, and (▨) indicates average thickness after continuous treatment with BC (5 μg ml^{-1}). The bars indicating the average thickness values within a different letter group(s) are significantly different from each other at $P < 0.05$. The error bars indicate the standard error of the mean.

re-growth; 23 ± 1 μm) than for continuously-treated biofilms (5% re-growth) when BC-stress was relieved for 24 h. When intermittently-treated biofilms were continuously-treated with BC (5 μg ml^{-1}), the thickness of the biofilms increased to 34 ± 1 μm (72% increase) compared with the thickness of 37 ± 1 μm (66% increase) for continuously-treated biofilms at 216 h.

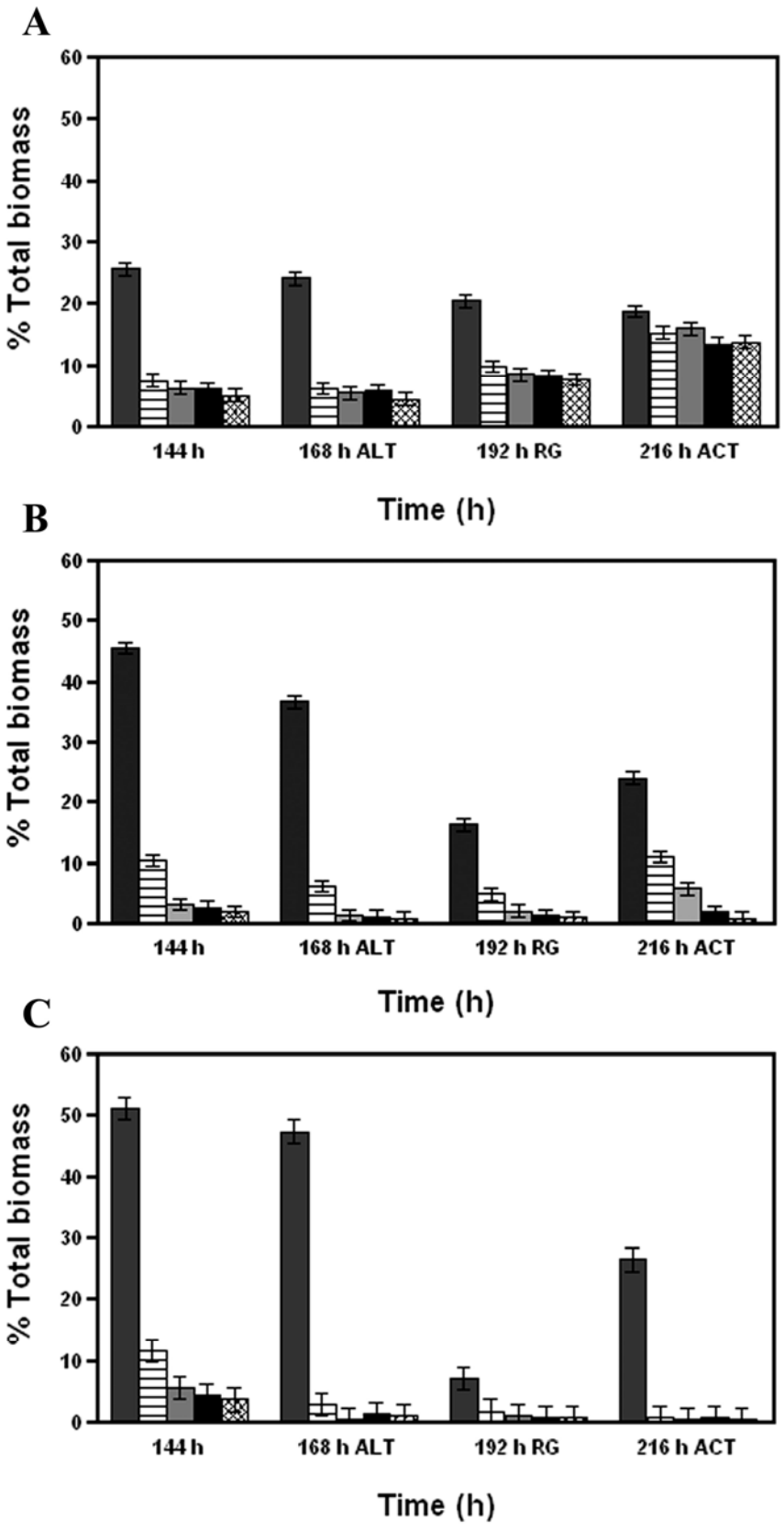
5.4.2. Effect of BC adaptation and lethal challenge on biofilm biomass

The amount of total biomass at the 5 optical sectioning depths remained constant in the case of continuously-treated biofilms, even following lethal BC challenge (Figure 5.4.3A). A significant increase ($P < 0.05$) in the abundance of total biomass at each sectioning depth was observed at 216 h, after 24 h continuous sub-lethal treatment with 5 μg ml^{-1} BC. For intermittently-treated biofilms (Figure 5.4.3B), there was only a marginal increase in the total biomass quantified at each of the 5 OTS compared with the untreated control biofilms (Figure 5.4.3C) after the 24 h recovery period following the lethal challenge. However, after 24 h of continuous BC treatment (5 μg ml^{-1}), the increase in total biomass of the intermittently-treated biofilms due to re-growth was significantly greater ($P < 0.05$) than seen for the untreated control biofilms, especially at the 3.7, 7.4, 11.1, and 14.8 μm OTS depths.

5.4.3. Effect of BC adaptation and lethal challenge on biofilm viability

After 144 h of continuous BC treatment, the viable biomass at the biofilm-substratum interface (0 μm OTS) represented 31% of the total field area, whereas the viable biomass present for biofilms subjected to intermittent BC treatment was 45%. The viability values for both systems (Figure 5.4.4A and B) decreased to less than 1% of total field area at all measured depths and locations in biofilms following lethal treatment, along with a proportional increase in the non-viable biomass. Notably, both adapted biofilms were able to re-grow (5% viable biomass at 0 μm OTS) when BC-stress was relieved for 24 h. In contrast, re-growth was minimal (1% viable biomass at 0 μm OTS) in the case of untreated control biofilms (Figure 5.4.4C). When continuously exposed to sub-lethal concentration of BC (5 μg ml^{-1}) for 24 h, the intermittently-treated biofilms exhibited no significant change ($P > 0.05$) in the abundance of viable biomass,

Figure 5.4.3. (facing page) The effect of BC treatments on the abundance of total biomass of (A) continuously-treated biofilms, (B) intermittently-treated biofilms, and (C) untreated control biofilms, as determined by fluorescein exclusion using CLSM and image analyses. The percentage total biomass at each time interval and at each OTS depth is the average of 15 measurements made at random biofilm locations. The 0-µm OTS represents the biofilm-substratum interface. ALT, the percentage total biomass at 168 h immediately after lethal BC treatment (500 µg ml^{-1}); RG, the percentage total biomass 24 h after lethal treatment; ACT, the percentage total biomass after 24 h continuous treatment with BC (5 µg ml^{-1}). The symbols (▬), (▭), (▬), (▬), and (▩) indicate the percentage total biomass at 0, 3.7, 7.4, 11.1, and 14.8 µm OTS depths, respectively. The error bars indicate the standard error of the mean.


A
% Total biomass
60
50
40
30
20
10
0
144 h
168 h ALT
192 h RG
216 h ACT
Time (h)
B
% Total biomass
60
50
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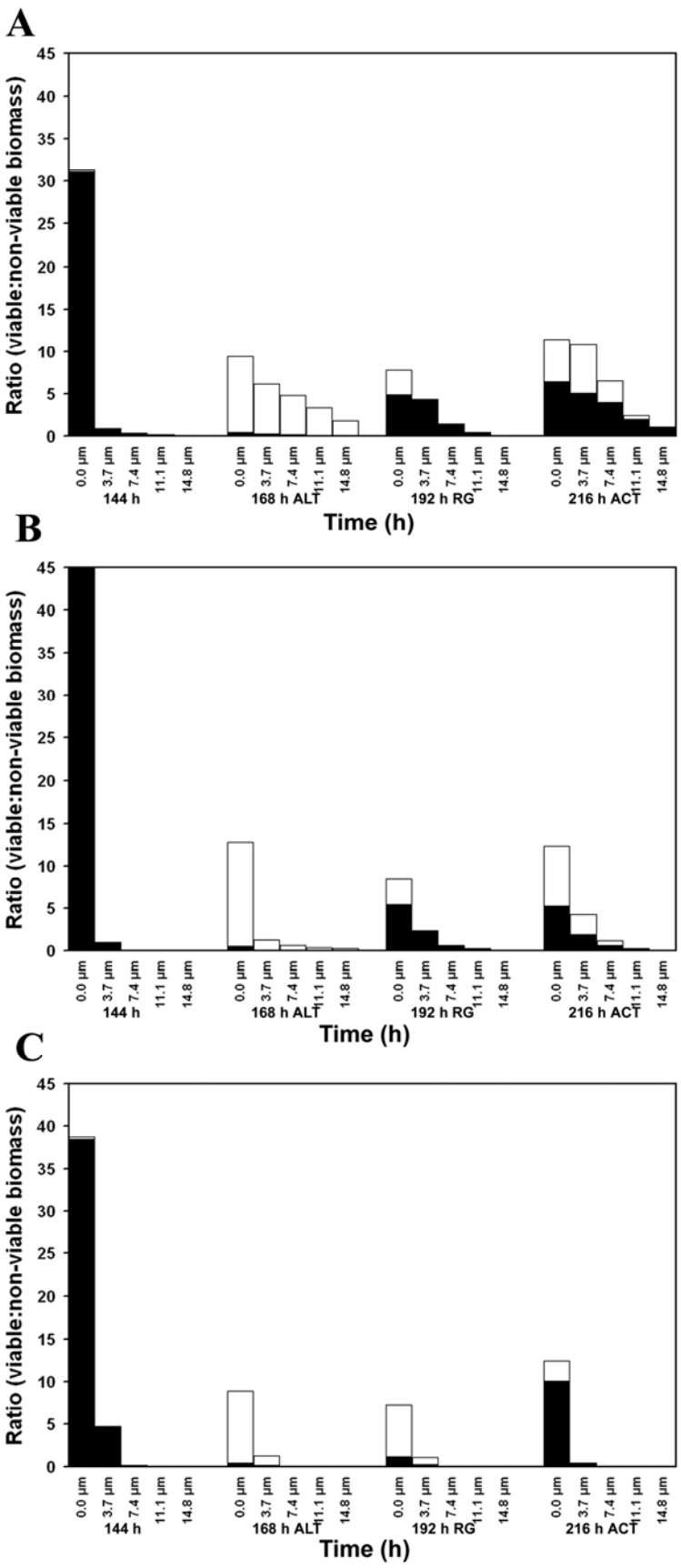
10
0
144 h
168 h ALT
192 h RG
216 h ACT
Time (h)
C
% Total biomass
60
50
40
30
20
10
0
144 h
168 h ALT
192 h RG
216 h ACT
Time (h)

Figure 5.4.4. (facing page) The effect of BC treatments on the viable biomass to non-viable biomass ratio in (A) continuously-treated biofilms, (B) intermittently-treated biofilms, and (C) untreated control biofilms, as determined by staining with *Bac*Light™ Viability Probe and quantified by CLSM and image analyses. The abundance of viable and non-viable biomass for each time interval and at each OTS depth is the average of 15 measurements made at random biofilm locations. The 0-μm OTS represents the biofilm-substratum interface. ALT, the abundance of viable and non-viable biomass at 168 h immediately after lethal BC treatment (500 μg ml^{-1}); RG, percentage viable or non-viable biomass 24 h after lethal treatment; ACT, the abundance of viable and non-viable biomass after continuous treatment with BC (5 μg ml^{-1}). The symbols (▬) and (▭) indicate the abundance of viable and non-viable biomass, respectively.


A
B
C
Ratio (viable:non-viable biomass)
0
5
10
15
20
25
30
35
40
45
0.0 µm
3.7 µm
7.4 µm
11.1 µm
14.8 µm
144 h
168 h ALT
192 h RG
216 h ACT
Time (h)

whereas significant re-growth ($P < 0.05$) in viable biomass was noticed for biofilms adapted to BC by continuous exposure.

5.4.4. Protein expression of BC-adapted biofilms

The expression of proteins in BC-adapted biofilms was compared with the protein expression profile of 168 h untreated control biofilms (Figure 5.4.5; Tables 5.4.1 and 5.4.2); 90 and 82 protein spots were detected in adapted and untreated control biofilms, respectively. Of the 40 protein spots examined, 22 proteins were up-regulated in both biofilm systems adapted to BC. However, 18 proteins were down-regulated in BC-adapted biofilms. The major proteins up-regulated in both BC-adapted biofilms included those involved in the degradation of 1,2-propanediol (1,2-PD) (PduJ and PduA), energy metabolism (TpiA and Eno), amino acid biosynthesis (WrbA), protein biosynthesis (RplL, Tsf, Tuf, DsbA, TrxA, and RpoZ), nutrient-binding (FruB), detoxification (Tpx, SodB, and probable peroxidase STY0440 (putative thiol-alkyl hydroperoxide reductase)), and adaptation (CspA). Some hypothetical proteins (GntY, YnaF, YcbL, and YjgF), and a putative periplasmic protein were also found to be up-regulated. Down-regulated proteins were those involved in ATP synthesis (AtpA) degradation (GarR), energy metabolism (GpmA, FbaA, GapA, RpiA, Mdh, and AceK), adaptation (UspA), broad regulatory functions (Hns), proteolysis (DegQ), cell envelope formation (RfbH), nutrient-binding (ArtI, MglB, and OppA), and the chaperone protein Hsp70 (DnaK).

5.5. Discussion

Salmonella serovar Enteritidis biofilms were observed to become adapted to BC by intermittent or continuous exposure to sub-lethal concentrations of the agent. Preliminary experiments were conducted to examine the effect of different concentrations of BC on biofilms. Rapid erosion and loss of biomass was observed when biofilms grown for 48 h were continuously exposed to 10 μg ml^{-1} BC (Figure 5.4.1). Thus, a BC concentration of 1 μg ml^{-1}, which resulted in the least impact on biofilm thickness relative to the control, was chosen for a continuous treatment regimen. The thickness of biofilms exposed to continuous or intermittent BC-treatment was

Figure 5.4.5. (facing page) Total proteins of p*I* values ranging from 4 and 7, which were differentially-expressed in biofilms adapted to BC. The images illustrate the representative 2D-PAGE gels pertaining to proteins extracted from (A) biofilms adapted to continuous treatment of BC (1 μg ml^{-1}), (B) biofilms adapted to intermittent treatment of BC (10 μg ml^{-1} for 10 min daily), and (C) untreated control biofilms. The description of the proteins and their levels of expression are illustrated in Table 5.4.1 (up-regulation) and Table 5.4.2 (down-regulation). The symbol (□) indicate the location of the protein spot in the control; the symbol (△) indicates up-regulation, and (▽) indicates down-regulation of the protein relative to the expression in untreated control biofilms.

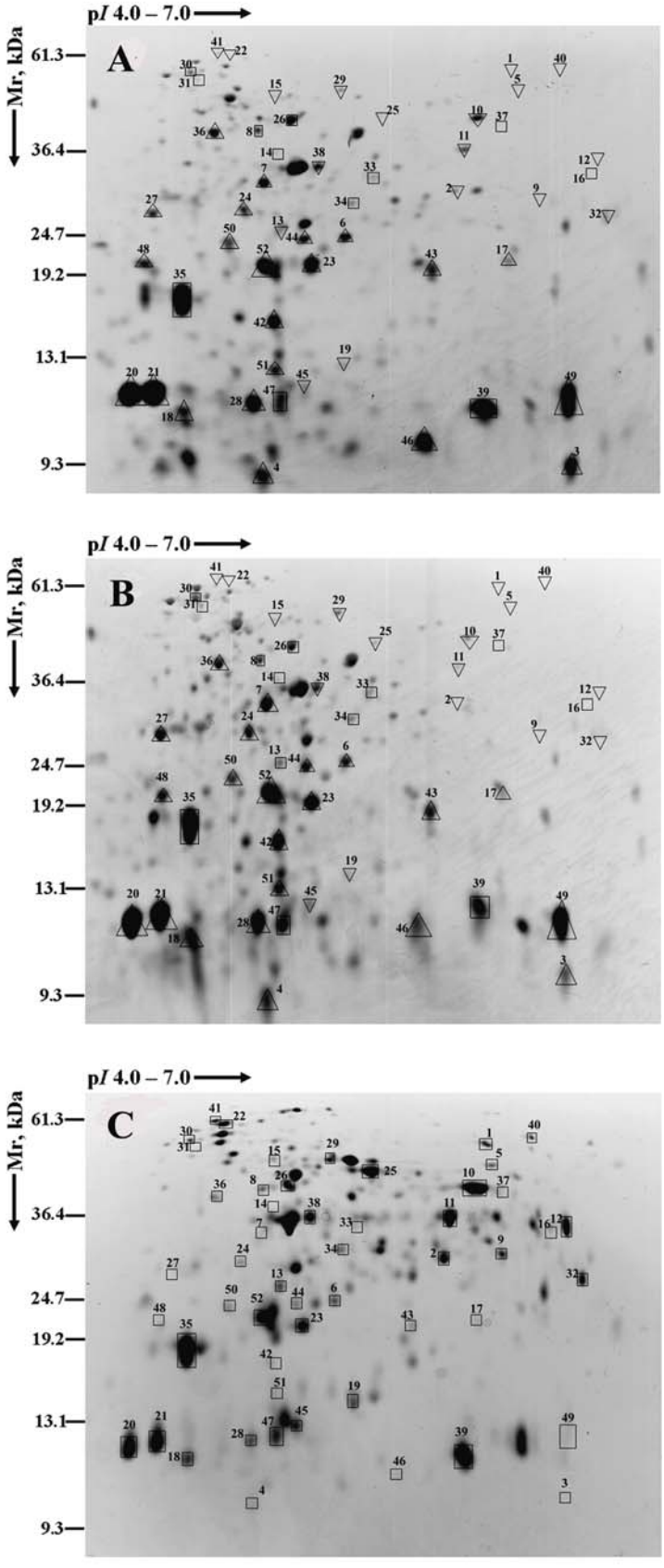

pI 4.0 – 7.0
Mr, kDa
61.3
36.4
24.7
19.2
13.1
9.3
A
B
C

Table 5.4.1. Proteins up-regulated in *Salmonella* serovar Enteritidis biofilms grown for 168 h and exposed to sub-lethal concentrations of BC

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold increase[b]	
						Biofilms continuously-treated with BC	Biofilms intermittently-treated with BC
Degradation (carbon compounds)	1	*pduJ*	Putative propanediol utilization protein J	9.07	6.50	84.76	40.72
	2	*pduA*	Putative propanediol utilization protein A	9.59	6.72	5.25	13.00
Energy metabolism (glycolysis)	3	*tpiA*	Triose-phosphate isomerase	26.92	5.68	3.27	3.02
	4	*eno*	Enolase	45.47	5.25	36.54	61.71
Aromatic amino acid biosynthesis	5	*wrbA*	Flavoprotein WrbA	20.74	5.79	8.10	25.43
Biosynthesis of cofactors, prosthetic groups, and carriers	6	*trxA*	Thioredoxin 1	11.68	4.67	1.86	3.57
Ribosomal protein synthesis and modification	7	*rplL*	50S Ribosomal subunit proteins L7 and L12	12.17	4.60	1.78	1.92
	8	*rplL*	50S Ribosomal subunit proteins L7 and L12	12.17	4.60	1.87	1.78
Protein translation and modification	9	*tsf*	Protein chain elongation factor Ts	30.36	5.13	8.81	8.65
	10	*tuf*	Elongation factor Tu (Fragment)	29.29	5.30	41.83	63.09
	11	*dsbA*	Thiol:disulfide interchange protein	22.91	5.64	2.48	2.39
RNA synthesis, RNA modification, and DNA transcription	12	*rpoZ*	DNA-directed RNA polymerase ω-chain	10.24	4.87	4.61	5.32
Cell processes (carbohydrate binding proteins)	13	*fruB*	Phosphotransferase system enzyme II (Fructose-specific IIA/FPr component)	39.59	4.87	4.17	4.36

Continued…

Table 5.4.1. continued.

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold increase[b]	
						Biofilms continuously-treated with BC	Biofilms intermittently-treated with BC
Cell processes (detoxification)	14	*tpx*	Thiol peroxidase	17.98	4.75	36.82	39.68
	15	NK[c]	Probable peroxidase STY0440	22.32	5.24	6.75	7.49
	16	*sodB*	Superoxide dismutase [Fe]	21.18	5.58	27.13	19.81
Adaptation and atypical conditions	17	*cspA*	7.4-kDa cold-shock protein	7.27	5.57	110.13	102.63
Hypothetical proteins	18	*gntY*	Hypothetical protein GntY	20.94	4.52	30.70	11.98
	19	*ynaF*	Conserved hypothetical protein STY1416 (putative universal stress protein)	15.70	5.93	2.06	1.95
	20	*ycbL*	Hypothetical protein YcbL (putative metallo-*β*-lactamase)	23.73	4.95	6.27	7.08
	21	*yjgF*	Conserved hypothetical protein YjgF	13.57	5.36	33.81	48.42
Miscellaneous proteins	22	NK[c]	Putative periplasmic protein (*Salmonella* serovar Typhi strain CT18)	21.44	NK[c]	2.86	2.66

Mr, molecular mass; p*I*, isoelectric point.
[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.
[b] Fold increase in protein expression from that of untreated control biofilms.
[c] NK, Not known.

Table 5.4.2. Proteins down-regulated in *Salmonella* serovar Enteritidis biofilms grown for 168 h and exposed to sub-lethal concentrations of BC

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold decrease[b]	
						Biofilms continuously-treated with BC	Biofilms intermittently-treated with BC
Degradation (carbon compounds)	23	*garR*	Tartronate semialdehyde reductase	30.73	5.59	0.09	0.03
Degradation (proteins, peptides, and glycoproteins)	24	*degQ*	Serine endoprotease	47.28	6.80	0.28	0.33
Energy metabolism (ATP synthesis)	25	*atpA*	ATP synthase α-subunit	54.98	5.80	0.13	0.13
Energy metabolism (glycolysis)	26	*gpmA*	Phosphoglycerate mutase 1	28.36	5.78	0.02	0.05
	27	*fbaA*	Fructose 1,6-bisphosphate aldolase Class II	39.36	5.58	0.18	0.04
	28	*gapA*	Glyceraldehyde 3-phosphate dehydrogenase A	35.46	6.32	0.11	0.01
Energy metabolism (tricarboxylic acid cycle)	29	*mdh*	Malate dehydrogenase	32.48	6.01	0.02	0.02
Energy metabolism (non-oxidative phase of pentose phosphate pathway)	30	*rpiA*	Ribose-5-phosphate isomerase A	22.90	5.08	0.42	0.68
Energy metabolism (glyoxylate bypass)	31	*aceK*	Isocitrate dehydrogenase kinase/phosphatase	46.09	5.99	0.48	0.66
Broad regulatory functions	32	*hns*	DNA-binding protein H-NS (histone-like protein II)	15.41	5.32	0.11	0.16

Continued…

Table 5.4.2. continued.

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold decrease[b]	
						Biofilms continuously-treated with BC	Biofilms intermittently-treated with BC
Ribosomal protein synthesis and modification	33	*rpsA*	30S Ribosomal subunit protein S1	61.25	4.89	0.08	0.05
Protein translation and modification	34	*tufA*	Translation elongation factor (EF-Tu.A)	43.40	5.30	0.25	0.25
Cell envelope (lipopolysaccharides)	35	*rfbH*	Lipopolysaccharide biosynthesis protein	48.10	5.27	0.06	0.02
Cell processes (amino acid binding proteins)	36	*artI*	Arginine-binding periplasmic protein 1 precursor	27.00	7.66	0.24	0.03
Cell processes (carbohydrate binding proteins)	37	*mglB*	D-galactose/D-glucose-binding periplasmic protein	35.81	5.81	0.57	0.44
Cell processes (binding proteins [other])	38	*oppA*	Oligo-peptide binding protein complexed with Kvk, chain A	58.81	5.85	0.09	0.09
Cell processes (chaperones)	39	*dnaK*	Chaperone protein Hsp70	69.13	4.83	0.17	0.15
Adaptation and atypical conditions	40	*uspA*	Universal stress protein A	15.95	5.12	0.18	0.18

Mr, molecular mass; p*I*, isoelectric point.
[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.
[b] Fold decrease in protein expression from that of untreated control biofilms.

significantly ($P < 0.05$) affected relative to that of the untreated control biofilms. Both BC treatment regimens negatively affected the thicknesses of biofilms prior to the lethal BC challenge. However, after the lethal challenge, both adapted biofilms grew significantly ($P < 0.05$) during the 24 h re-growth period in the BC-free environment, and thereafter during continuous sub-lethal (5 μg ml^{-1}) exposure to BC. The re-growth in terms of total biomass was also significantly greater ($P < 0.05$) in the case of BC-adapted biofilms. Interestingly, the amount of re-growth was significantly higher in continuously-treated biofilms than in intermittently-treated biofilms. The increase in the abundance of biofilm biomass per optical thin section depth was also significantly greater ($P < 0.05$) in biofilms continuously-treated with BC than in those where BC was applied intermittently. These results, along with a significant increase in viable biomass in the case of continuously-treated biofilms, suggests that adaptation via continuous exposure to BC results in greater BC resistance in biofilm cells than following intermittent BC adaptation regimens.

The assessment of the action of QACs on both bacterial planktonic cells (Langsrud and Sundheim, 1996) and biofilms (Luppens et al., 2002; Simões et al., 2005) has been reported. There have been reported discrepancies with respect to the proportion of actual viable count to the observed viable count of bacterial cells following exposure to BC (Langsrud and Sundheim, 1996). The results of this study also indicated that a single parameter could not be relied upon to assess the viability of bacterial biofilms exposed to antimicrobial agents. The measurement of total biomass alone was insufficient to provide conclusive information on the effect of BC lethality, since total biomass included the biomass of both viable and non-viable cells; non-viable cells were retained in the biofilm matrix after cell death following lethal BC treatment. However, increases in biofilm thickness and biomass due to re-growth are positive indicators of viability, and when combined with fluorescent probes sensitive to the integrity of the cellular semi-permeable membrane (viability), offer greater information.

There are reports on the disruption of outer membrane and leakage of intracellular contents in Gram-negative bacteria exposed to QACs (Maxcy et al., 1971; Simões et al., 2005). The viable biomass of both adapted biofilms was found to be significantly less ($P < 0.05$) in all the 5 OTS immediately after the lethal challenge,

indicating the death of cells. However, there was significant increase ($P < 0.05$) in the abundance of viable biomass after 24 h following lethal BC challenge because of re-growth. The lethal BC treatment might have caused only partial disruption of the outer membrane of the biofilm cells causing influx of propidium iodide; cells thus appeared as non-viable, although they may not have actually been dead. Inconsistencies in bacterial viability assessment using the *Bac*Light™ Live/Dead Viability Probe have previously been reported (Auty et al., 2001). Alternatively, it is also quite likely that only a small percentage of cells may have actually been alive; however, once the BC stress was relieved, cells were able to recover and multiply since they were adapted to BC. In general, the presence of "survivors" following antimicrobial treatment is a typical scenario often described as a key in the persistence and re-growth of biofilms. The "persister" cell theory (Lewis, 2001) also fits this model.

Analyses of the differential protein expression patterns of BC-adapted and BC-untreated control *Salmonella* serovar Enteritidis biofilms was used to examine molecular mechanisms of adaptive resistance (Tables 5.4.1 and 5.4.2). The similarity in the proteome patterns of biofilms adapted to BC by continuous and intermittent treatment regimens provides strong evidence that essentially the same metabolic pathways were employed during the process of adaptation (Figure 5.4.5). Furthermore, the similar patterns of specific proteins and their levels of expression between the two adapted biofilms validate the reproducibility of the methods and techniques used. A difference in the protein expression pattern can clearly be ascertained by comparing the protein expression profiles of BC-adapted and untreated control biofilms, a consequence of either exposure or adaptation to BC. In BC-adapted biofilms, there was significant down-regulation ($P < 0.05$) of high molecular weight proteins (> 25 kDa) of p*I* 5.6 to 7, and a significant up-regulation ($P < 0.05$) of all other proteins. It is hypothesized from these observations that the up- and/or down-regulation of the clusters of proteins with similar physical and biochemical properties (pH, molecular weight, etc.) was essential in the adaptive resistance to BC and homeostasis of biofilms.

Various enzymes involved in cold-shock response, stress response, and detoxification were significantly up-regulated in the adapted biofilms, along with an overall increase in cellular protein biosynthesis (Table 5.4.1). The up-regulation of a

battery of defense enzymes, including (periplasmic) thiol peroxidase (Tpx), superoxide dismutase [Fe] (SodB), and a probable peroxidase participating in the destruction of toxic radicals formed in the cytoplasmic membrane and within the cells was observed in adapted biofilms. Various neutralizing enzymes, including, peroxidases, superoxide dismutases, catalases, glutathione reductase, alkyl hydroperoxide reductases, and DNA repair enzymes (e.g., exonuclease III) have been characterized as defense enzymes in *Salmonella* and *E. coli* (Farr and Kogoma, 1991; McDonnell and Russell, 1999; Clöete, 2003). The oxidant-degrading enzymes (Tpx and SodB) are usually up-regulated during oxidizing stress response leading to resistance within hours of exposure to sub-lethal concentration of oxidizing biocides (Clöete, 2003). The role of these defense enzymes in BC-adapted biofilms is not clear, but may indicate that following BC-induced membrane damage, secondary oxidizing stresses may follow.

Among the proteins participating in adaptation and atypical conditions, the 7.4 kDa cold-shock protein (CspA) was significantly (>100-fold) up-regulated. CspA is involved in the cold-shock response by binding to, and stimulating the transcription of cold-shock-inducible promoters of *hns* and *gyrA*. In contrast, the universal stress protein (UspA), which confers resistance to DNA-damaging agents as well as a variety of other stresses including nutrient depletion and starvation, was significantly down-regulated (>5-fold) in adapted biofilms. The chaperone protein Hsp70 (DnaK) was also significantly down-regulated (~6-fold) in adapted biofilms, suggesting that UspA and DnaK were not significantly expressed in BC-adapted biofilms. Conversely, the conserved hypothetical protein (YnaF), a putative universal stress protein, was up-regulated (~2-fold) in adapted biofilms, thus an alternate pathway of stress response may be utilized by the adapted biofilm cells. This may further suggest that "adapted" biofilms do not require UspA and DnaK, perhaps having up-regulated these during an initial response following BC exposure. The "pleiotropic regulator" DNA-binding protein H-NS (Hns), with broad regulatory functions, was found to be significantly down-regulated (>6-fold) in BC-adapted biofilms. Hns binds tightly to ds-DNA, increases its thermal stability and inhibits transcription (Pon et al., 1988). Thus, CspA involved in the cold-shock response might not act through *hns* in *Salmonella* serovar Enteritidis biofilms. It also strengthens the hypothesis that the exposure of biofilms to BC leads only to cold-

shock response, and not heat-shock response. The response of biofilms to BC is suggested to be rather specific, since the cells elicited only the cold-shock response. It was also observed that there was up-regulation of CspA in BC-adapted planktonic cells (*see* Figure 6.4.5; Table 6.4.4). CspA has been reported to function as an RNA chaperone in *E. coli* (Jiang et al., 1997), and certain inhibitors of translation (e.g., chloramphenicol, tetracycline, erythromycin, fusidic acid, and spiramycin) resulted in the induction of the cold-shock response – the induction of cold-shock proteins, repression of heat-shock proteins, and continued synthesis of transcriptional and translational proteins (VanBogelen and Neidhart, 1990; Jones and Inouye, 1994). Thus, cold-shock response might be implicated as a mechanism of cross-resistance to chloramphenicol and tetracycline in BC-adapted *Salmonella* and *E. coli* cells, as reported previously (Braoudaki and Hilton, 2004).

BC-adapted biofilms also underwent a significant up-regulation of PduJ and PduA (>40-fold and >5-fold, respectively), proteins involved in 1,2-propanediol (1,2-PD) utilization. *S. enterica* utilizes 1,2-PD as a carbon and energy source in an adenosyl-B_{12}-dependent fashion (Jeter and Roth, 1987). The 1,2-PD utilization (*pdu*) locus in *Salmonella* serovar Typhimurium is comprised of 23 genes, coding for enzymes and at least 15 structural proteins participating in the 1,2-PD catabolism (Bobik et al., 1999; Havemann and Bobik, 2003). *S. enterica* forms polyhedral organelles (*S. enterica* organelles), and this organelle function to convert 1,2-PD to propionyl CoA and to minimize aldehyde toxicity. 1,2-PD catabolism has also been identified as the primary reason for *de novo* cobalamin (B_{12}) biosynthesis in *S. enterica* (Havemann and Bobik, 2003). Interestingly, the strong up-regulation of PduJ and PduA observed in BC-adapted biofilms suggests that this organism utilized the 1,2-PD utilization pathway while growing in the presence of BC, perhaps for energy generation, degradation of BC, minimizing aldehyde toxicity, or for cobalamin biosynthesis.

Intracellular accumulation of proteins might also contribute to the adaptive resistance to BC. The *de novo* synthesis of protein as a mechanism of increased thermotolerence and resistance to trisodium phosphate has been hypothesized previously in *Salmonella* serovar Enteritidis (Sampathkumar et al., 2004). Some of the components of cellular protein biosynthetic machinery (ribosomal subunit proteins, protein chain

elongation factors, and amino acid biosynthetic protein) were significantly up-regulated in adapted biofilms. Thioredoxin 1 (TrxA), ribosomal subunit proteins L7 and L12 (RplL), protein chain elongation factors (Tsf and Tuf), thiol:disulfide interchange protein (DsbA), and DNA-directed RNA polymerase ω-chain (RpoZ) were up-regulated in their level of expression in adapted biofilms. Significant up-regulation of WrbA (> 8-fold), involved in the aromatic amino acid biosynthesis was also noticed. Serine endoprotease (DegQ) involved in degrading SsrA-tagged proteins was down-regulated (~3-fold) in the biofilms. Similarly, the pretreatment of *Salmonella* and *E. coli* with sub-inhibitory doses of hydrogen peroxide has been shown to induce catalase and glutathione reductase, as well as other nonessential proteins that accumulate to protect the cells (Storz and Altuvia, 1994; Mukhopadhyay and Schellhorn, 1997). The hypothetical proteins, GntY, YcbL, and YjgF were significantly up-regulated in both BC-adapted biofilms. GntY is thought be involved in gluconate metabolism. YcbL is suggested to be involved in phosphorylation and sensory transduction. The putative periplasmic protein which has been significantly up-regulated (> 2.6-fold) in the biofilms might function as an efflux protein or degradative enzyme.

In conclusion, BC-adapted *Salmonella* serovar Enteritidis biofilms acquired the ability to survive normally-lethal exposure to BC, and then re-grow. While there were differences in the growth responses of biofilms continuously- and intermittently-treated with BC, it is significant that either route of adaptation resulted in similar protein expression patterns. BC adaptation has relevance to the re-growth of pathogenic microorganisms on surfaces in clinical settings and food processing environments following routine sanitary procedures. Adaptation to BC occurred concurrently with the up-regulation of key proteins involved in cold-shock response, stress response, and detoxification, and an overall increase in protein biosynthesis. Thus, the up-regulation of these important proteins explains the mechanisms responsible for adaptive resistance to BC in *Salmonella* serovar Enteritidis biofilms.

5.6. Connection to the next study

The biofilms adapt to BC exposure via biofilm architectural changes and cellular physiological changes. The next study was performed to assess how planktonic cells

respond to a sub-lethal BC exposure. Since, the planktonic and biofilm phases are phenotypically distinct, it was intriguing as to see how the two phenotypes respond to a similar stress of sub-lethal BC exposure.

6. DIFFERENTIAL ADAPTIVE RESPONSE AND SURVIVAL OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS PLANKTONIC AND BIOFILM CELLS EXPOSED TO BENZALKONIUM CHLORIDE

6.1. Abstract

This study reports the functional difference in the adaptive response and survival of planktonic and biofilm phenotypes of *Salmonella enterica* serovar Enteritidis adapted to benzalkonium chloride (BC). Both planktonic cells and biofilms were continuously exposed to 1 μg ml^{-1} of BC for 144 h. The proportion of BC-adapted biofilm cells that survived a lethal BC concentration (30 μg ml^{-1}) was significantly higher (4.6-fold) than that of BC-adapted planktonic cells. Similarly, the proportion of survivors among the BC-adapted biofilm cells was significantly higher (18.2-fold) than the survivors among BC-adapted planktonic cells (3-fold) at lethal BC concentration, when compared with their respective untreated (without BC) counterparts. A significantly higher ($P < 0.05$) proportion of survival was noticed among BC-adapted biofilm cells relative to BC-adapted planktonic cells following heat-shock at 55°C for 10 min. Cell surface roughness of biofilm cells was also significantly higher ($P < 0.05$) than that of planktonic cells. Fatty acid composition was significantly influenced by phenotype and BC adaptation. Key proteins up-regulated in BC-adapted planktonic and biofilm cells were CspA, TrxA, Tsf, YjgF, and a probable peroxidase STY0440 (putative thiol-alkyl hydroperoxide reductase). Nine and 17 unique proteins were up-regulated in BC-adapted

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This chapter is reproduced from a manuscript prepared for publication. The manuscript is co-authored by S. Vidović and D. R. Korber. All experimental works with the exception of fatty acid profile and mass spectrometry analyses were carried out by me. In addition, I wrote the initial draft of the manuscript. Minor modifications were made to the original version to maintain thesis format and style.

planktonic and biofilm cells, respectively. These results suggest that enhanced biofilm-specific up-regulation of 17 proteins, along with the increased expression of CspA, TrxA, Tsf, YjgF, and probable peroxidase as well as phenotype-specific alterations in cell surface roughness and shift in fatty acid composition acted synergistically, conferring enhanced survival to BC-adapted biofilm cell population, relative to their BC-adapted planktonic cell counterparts.

6.2. Introduction

Antimicrobial resistance of bacterial pathogens has been attributed either to inherited or non-inherited resistance mechanisms (Levin and Rozen, 2006). Non-inherited, or adaptive, resistance to antibiotics and other chemotherapeutic agents has been widely reported in various species of bacteria; the physiological state and the physical structure of the adapted population have been attributed to this phenomenon, which is purely phenotypic in nature (Levin, 2004; Levin and Rozen, 2006). The exact biochemical mechanisms of adaptive resistance, and the cross-resistance of "adapted" strains to unrelated chemotherapeutic agents and biocides and *vice versa*, as well as the contribution of multi-drug resistant "superbugs" to the treatment failure remain largely unknown (Levy, 2001; Russell, 2003a; Braoudaki and Hilton, 2004; Levin, 2004; Sheldon, 2005).

Antimicrobial agents have been broadly classified as biocides (antiseptics, disinfectants, and preservatives) and antibiotics (Denyer and Maillard, 2002). It is generally believed that unlike antibiotics, bacteria rarely become resistant to biocides because of their broad activity spectrum and numerous target sites (Braoudaki and Hilton, 2005). Typically, antibiotics have only one major target site of action, a feature known to facilitate the development of bacterial resistance through mutational events (Russell, 2003b; Braoudaki and Hilton, 2005). Various molecular mechanisms have been reported to play roles in the bacterial adaptive responses to antimicrobial compounds. These adaptive mechanisms act either singly or synergistically in order to bestow resistance; among them, "slow" multiplication resulting in "persisters", SOS response that blocks cell division during the repair of DNA damage, cold-shock response, stress response, detoxification, altered permeability of the outer membranes due to changes in

lipopolysaccharide (LPS) composition, outer membrane proteins, cytoplasmic membrane proteins, fatty acid composition and content of cytoplasmic membrane, cell surface charge, hydrophobicity, active efflux of the agent etc. are believed to be significant (Karlowsky et al., 1996; Bianchi and Baneyx, 1999; Spoering and Lewis, 2001; Loughlin et al., 2002; Braoudaki and Hilton, 2005; Levin and Rozen, 2006; Mangalappalli-Illathu and Korber, 2006). It has been reported that a single microorganism may have multiple, possibly interconnected, adaptive mechanisms depending on the nature of the antimicrobial agent (Campanac et al., 2002; Szomolay et al., 2005). There are also recent reports suggesting that molecular mechanisms providing bacterial resistance to biocides may provide cross-protection against certain antibiotics (Braoudaki and Hilton, 2004; Langsrud et al., 2004; Mangalappalli-Illathu and Korber, 2006; Bore et al., 2007).

Quaternary ammonium compounds (QAC) are a major group of cationic surface-active antimicrobial agents; acquired resistance to these compounds in bacterial pathogens such as *E. coli*, *P. aeruginosa*, and *S. enterica* serovars has been reported (Maxcy et al., 1971; Sakagami et al., 1989; Braoudaki and Hilton, 2005). Gram-negative bacteria are considered to be more resistant to QACs than Gram-positive bacteria. Benzalkonium chloride (BC) is a well-known member of this group, which has been widely used as a surface disinfectant, antiseptic, and preservative. QACs are bacteriostatic in low concentrations and bactericidal in high concentrations; the antibacterial activity is associated with adsorption by the bacterial cells and subsequent leakage of cellular constituents (Maxcy et al., 1971). Sakagami et al. (1989) reported that the ability of BC to permeate BC-resistant *P. aeruginosa* was reduced because of the increases in cellular fatty acids such as phospholipids as well as fatty and neutral lipids in the cell wall. The development of adaptive resistance to the antibiotic, erythromycin and various antimicrobial agents such as BC, chlorhexidine, and triclosan has been reported in *Salmonella* serovars Enteritidis, Typhimurium, and Virchow (Braoudaki and Hilton, 2004, 2005; Mangalappalli-Illathu and Korber, 2006).

It has been construed that the biofilm cells respond in a significantly different manner to the antimicrobial agents than their planktonic counterparts. Mikkelsen et al. (2007) reported that protein profiles of biofilms were found to more closely resemble

those of exponentially growing planktonic cells than those of planktonic cells in the stationary phase. Moreover, there is evidence that proteins involved in oxidative stress response, cell envelope synthesis, as well as synthesis of exopolymeric substances (EPS) become up-regulated in biofilms, indicating that these factors might contribute to cell survival, persistence, and growth in a biofilm environment (Resch et al., 2005). Therefore, subsequent to prolonged exposure to antimicrobial agents, this could result in significant differences in the adaptive responses of planktonic and biofilm cells. Physiologic or phenotypic adaptation resulting in "biocide tolerance" has been attributed to biofilms (Donlan and Costerton, 2002; Sheldon, 2005). Biofilm resistance to biocides may result from slow microbial growth rates that are attributable to nutrient depletion within biofilms, binding of the biocide to the EPS of biofilms, neutralization or degradation of the biocide, as well as the expression of biofilm-specific phenotypes (Sheldon, 2005; Szomolay et al., 2005). The EPS also protect biofilm bacteria from immunological processes such as opsonization and phagocytosis (Jensen et al., 1990; Costerton et al., 1999).

The planktonic and biofilm cells of *E. coli*, *P. aeruginosa*, *P. pseudomallei*, *S. aureus*, and *S. sanguis* were previously shown to exhibit some major differences in the minimum inhibitory concentrations (MIC) of various antibiotics to which they were susceptible (Donlan and Costerton, 2002). The lethal effect of chlorine on planktonic and biofilm cells of *Salmonella* spp. was compared and found that there was complete lethality of planktonic cell populations, whereas the reduction in the biofilm cell population was less than 1 $\log_{10}$ unit (Joseph et al., 2001). It was further reported that *Salmonella* serovar Typhimurium biofilm cells were significantly more resistant to chlorination than planktonic cells; the biofilm matrix has been believed to provide protection against reactive sodium hypochlorite (Scher et al., 2005). However, there also exist contradicting reports on the enhanced ability of the biofilm phenotype to resist killing. There is a report that biofilms and planktonic cells of *P. aeruginosa* had similar resistance to killing by different antibiotics (Spoering and Lewis, 2001). Thus, there are various contributing factors such as species and strain of bacteria as well as the type and concentration of antimicrobial agent that determine the susceptibility of bacteria to killing or the development of adaptive resistance.

Bacteria employ an array of survival mechanisms to become adapted and to survive lethal effects of antimicrobial compounds. This study investigated various cellular responses of *Salmonella enterica* serovar Enteritidis planktonic and biofilm cells that contribute to adaptive resistance and survival against BC in qualitative and quantitative terms, highlighting the significance of phenotype-specific responses contributing to enhanced survival. The study examined: (i) the survival in lethal BC environment and (ii) to heat-shocking, (iii) surface roughness analyses using atomic force microscopy (AFM) and image analyses, (iv) fatty acid composition by fatty acid methyl esters (FAME) analyses, and (v) differential protein expression by means of two dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of BC-adapted and untreated planktonic and biofilm phenotypes of this foodborne enteric pathogen.

6.3. Materials and methods

6.3.1. Media and chemicals

Tryptic Soy Agar (TSA), Standard Plate Count Agar (SPCA), and Trypticase Soy Broth (TSB) were purchased from BBL (Becton Dickinson, Cockeysville, MD); benzalkonium chloride (BC; $C_6H_5CH_2N(CH_3)_2RCl$ (R = C_8H_{17} to $C_{18}H_{37}$)), magnesium chloride ($MgCl_2$), phenylmethylsulphonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), DNase, RNase A, bromophenol blue, DL-dithiothreitol (DTT), and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO); sodium chloride was from EM Science (Gibbstown, NJ); EDTA was from J. T. Baker Chemical Co. (Philipsburg, NJ); glycerol, sodium dodecyl sulphate (SDS), Tris base, and urea were purchased from Life Technologies (Grand Island, NY); and immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0), Immobiline DryStrip gels, and PlusOne^{TM} Protein Silver Staining Kit were purchased from GE Healthcare Bio-Sciences Inc. (Baie d'Urfé, QC, Canada).

6.3.2. Bacteria and culture conditions

Salmonella enterica serovar Enteritidis ATCC 4931 (hereafter referred to as *Salmonella* serovar Enteritidis) was cultured from a frozen stock on TSA plates

overnight at 37°C. Cells in the mid-log phase of growth were obtained by transferring a loopful of colony material from overnight TSA plates to 50 ml of 10% [wt/vol] TSB in an Erlenmeyer flask, and then incubating on a gyratory shaker (150 ± 5 rpm) held at room temperature (RT; 21 ± 2°C) for approximately 12 h. These cells, which were determined previously to be in the mid-log phase of growth (Mangalappalli-Illathu and Korber, 2006), were inoculated to cultivate planktonic and biofilm cells (*see* below).

6.3.3. Determination of minimum inhibitory concentration (MIC) and lethal concentration of BC

The MIC of BC for planktonic cells of *Salmonella* serovar Enteritidis was determined to be 15 μg ml^{-1} by doubling dilution-based nephalometry coupled with plating of mid-log phase planktonic cells treated with increasing concentrations of BC. A sub-lethal concentration of 1 μg ml^{-1} was selected for continuous BC application. The concentration of BC for lethal challenge was determined as 500 μg ml^{-1} BC, based on the preliminary observations and recommendations of regulatory agencies for surface disinfection using QACs (Health Canada, 1999; Food and Agriculture Organization, 2003).

6.3.4. Adaptation of *Salmonella* serovar Enteritidis planktonic cells to BC

Planktonic cells were grown in an Erlenmeyer flask held on a gyratory shaker (150 ± 5 rpm) continuously cultured at RT for 168 h in 175 ml of 10% [wt/vol] TSB; medium was continuously added and removed at a rate of 25 ml h^{-1}, resulting in a dilution rate of 0.14 h^{-1}. The medium was pumped into and out of the Erlenmeyer flask via silicone tubing using two Watson-Marlow peristaltic pumps (Model 202U; Watson-Marlow, Cornwall, UK). The medium in the flask was inoculated with 2 ml mid-log phase *Salmonella* serovar Enteritidis cells, prepared as outlined above, concentrated or diluted to an optical density (OD) equivalent to 0.5 McFarland standard (1.5 X 10^8 cfu ml^{-1}). After 24 h of growth, the planktonic cells were continuously exposed to a sub-lethal concentration (1 μg ml^{-1}) of BC over an additional 144 h of growth. Experiments using untreated planktonic cells grown under identical conditions (without BC) for a period of 168 h were used as control.

6.3.5. Adaptation of *Salmonella* serovar Enteritidis biofilms to BC

Biofilms were grown in multi-channel flow cells. The flow cells were constructed using polycarbonate sheets into which channels were milled, as described previously (Korber et al., 1994). Flow cell channels were sterilized by flushing with 5.25% [wt/vol] sodium hypochlorite solution for 10 min. Reservoirs of sterile nutrient medium (10% [wt/vol] TSB) were connected via silicone tubing to the flow cell channels and subsequently connected to the waste reservoir. Medium was pumped at a laminar flow velocity of 0.07 cm sec^{-1} through flow cells using a Watson-Marlow peristaltic pump. In this set up, the bulk flow rate of the nutrient medium through each flow cell channel was determined to be 25 ml h^{-1}. Each flow cell channel was individually inoculated with 0.5 ml mid-log phase *Salmonella* serovar Enteritidis cells (0.5 McFarland standard) (*see* above). Established (24 h) biofilms grown under laminar flow conditions were exposed continuously to a sub-lethal concentration of BC (1 µg ml^{-1}) over an additional 144 h of growth. Experiments using untreated control biofilms (without BC), which were grown under identical conditions for a period of 168 h, were also performed in the study.

6.3.6. Survival of BC-adapted and untreated cells in increasing lethal BC concentrations

BC-adapted and untreated planktonic and biofilm cells were collected and centrifuged at 4,000 rpm (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 3 min to pellet the bacterial cells. The supernatant was completely removed and the pellet resuspended in phosphate buffered saline (PBS; pH 7.2), thoroughly vortexed, and the cell density adjusted to an OD equivalent to 0.5 McFarland standard using a Biochrom Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, UK) set for measuring OD at 600 nm (OD_{600}). The bacterial suspension after serial dilution in PBS was used as the inoculum for the survival assay. The serially-diluted suspensions of BC-adapted and untreated planktonic and biofilm cells were immediately spread plated on SPCA containing increasing concentrations (0, 10, 20, 25, and 30 µg ml^{-1}) of BC. The time required to process cells (from pellet to the inoculation of SPCA plates) was ~10 min. The plates were incubated at 37°C for 24 h. The

proportion of survivors was determined by counting the colonies recovered on the SPCA plates containing increasing concentrations of BC (n = 4), and expressed in cfu ml^{-1}.

6.3.7. Treatment of BC-adapted and untreated cells with a lethal concentration (500 µg ml^{-1}) of BC

Suspensions of BC-adapted and untreated planktonic and biofilm cells were prepared in PBS as described above for the survival assay. Cell suspensions (10 ml) were treated with BC (final concentration of 500 µg ml^{-1}) and incubated at RT for up to 10 min. Aliquots (0.1 ml) were then collected at 2 min intervals until the end of the 10 min incubation period, serially-diluted using PBS at RT, and spread plated on SPCA. The plates were incubated at 37^{o}C for 24 h. The proportion of survivors on SPCA was expressed as cfu ml^{-1}.

6.3.8. Heat-shock experiments

Suspensions of BC-adapted and untreated planktonic and biofilm cells were prepared in PBS, as described above for the BC survival assay, and then incubated on waterbaths set at either 55 and 62.8^{o}C for 10 min. Aliquots (0.1 ml) were collected at 2 min intervals until the end of 10 min incubation period, serially-diluted using PBS at RT, and spread plated on SPCA. After incubation on SPCA for 24 h at 37^{o}C, the number of survivors was enumerated (n = 4), and expressed as cfu ml^{-1}.

6.3.9. Analysis of cell surface roughness using Atomic Force Microscopy (AFM)

Changes in cell surface roughness of BC-adapted (for 144 h) and untreated planktonic and biofilm cells were analyzed using AFM. The cell surface alterations following exposure to lethal concentrations of BC (30, 50, 100, and 500 µg ml^{-1} for 10 min) on BC-adapted and untreated control planktonic and biofilm cells were compared. Planktonic cells were allowed to air-dry on a coverslip for ~15 min, washed with PBS, and then rinsed with reverse osmosis (RO) water. Biofilm cells were prepared by removing pieces of coverslips from the flow cell channels and affixing the pieces onto a mounting coverslip using silicon adhesive. Subsequent washing was performed as for planktonic cells. Cells were lethally treated for 10 min using BC solutions (*see* above)

prepared in RO water, followed by washing with PBS and RO water. The samples were allowed to air-dry for ~15 min and placed in a humidifying chamber (RT and ~80% rH) until the AFM was performed (Cross et al., 2006; Del Sol et al., 2007).

AFM was performed using a Molecular Imaging PicoSPM I^{TM} with PicoScan 2100^{TM} controller and equipped with a piezo-scanner (Molecular Imaging Corp., Tempe, AZ). Imaging was carried out using oxidation-sharpened silicon nitride AFM probes with a tip radius of ca. 10 nm (cantilever nominal spring constant, k = 0.12 N m^{-1}; resonant frequency, 14 – 26 kHz) (DNP-S; Veeco Probes, Camarillo, CA) in the contact mode. The scanning force constant was ca. 3 nN and scan rates were ca. 1 Hz. Image areas of 0.5 X 0.5, 1 X 1, 5 X 5, and 20 X 20 μm^2 were collected from the same location in height and deflection modes simultaneously (Touhami et al., 2004; Pelling et al., 2005; Cross et al., 2006). Quantitative data were collected from the height mode images, whereas corresponding images for illustration purpose were collected in the deflection mode. Images were collected from samples derived from three independently replicated experiments.

This study followed the analytical method used by Cross et al. (2006) for determining bacterial cell surface roughness using AFM. The stored images (5 X 5 μm^2) were analyzed using PicoScan^{TM} software version 5.3.1a (Molecular Imaging Corp.) for determining the cell surface roughness. An area of 0.25 X 0.25 μm^2 was selected on the middle region of each cell image and then analyzed to determine average surface roughness of the region of the cell. The data obtained from 60 randomly selected cells (i.e., 20 cells each from three independently replicated experiments) were statistically analyzed to obtain cell surface roughness pertaining to the treatment, and expressed in Å.

6.3.10. Fatty acid profile analyses

FAME analyses were conducted as described previously (Annous et al., 1997; Sampathkumar et al., 2004) using 40 to 50 mg of washed BC-adapted and untreated planktonic and biofilm cell pellets. A gas-liquid chromatograph (Model 5890 series 2; Hewlett Packard, Avondale, PA) equipped with a flame ionization detector and a 25 m X 0.22 mm methyl phenyl silicone fused silica capillary column (Ultra 2; Hewlett Packard catalog # 19091B-102) was used for identification of FAMEs. Results were

automatically integrated by Hewlett Packard 3365 series II ChemStation software version A.03.21, and FAMEs were identified with the MIDI Microbial Identification Software (Sherlock TSBA Library version 4.1; Microbial ID, Inc., Newark, DE).

6.3.11. Protein expression analyses

BC-adapted and untreated planktonic and biofilm cells were collected after 168 h of growth and pelleted by centrifuging at 4,000 rpm (Model 5810 R with swing-bucket rotor A-4-81; Eppendorf, Hamburg, Germany) for 5 min. The cells were then disrupted and protein extracted. Total cellular proteins from biofilm and planktonic cells were electrophoretically separated by 2D-PAGE, as detailed previously (Mangalappalli-Illathu and Korber, 2006). Differentially-expressed proteins were then detected and quantified from the stored images of gels using Phoretix^{TM} 2D version 2004 analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). An increase in protein spot volume of 1.5-fold or more was interpreted as up-regulation, whereas a decrease in the spot volume of 1.5-fold or more was interpreted as down-regulation. Differentially-expressed proteins were identified by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis (Mangalappalli-Illathu and Korber, 2006). The LC-MS/MS data were processed using ProteinLynx software (Waters-Micromass) and searched against the NCBInr, MSDB, or Swiss-Prot/TrEMBL protein databases using Mascot Search (Matrix Science Ltd., London, UK). The biological function(s) of each protein identified were determined from the Wellcome Trust Sanger Institute and PUMA2 databases (Mangalappalli-Illathu and Korber, 2006).

6.3.12. Experimental replication and statistical analyses

All experimental data represents the average of at least three independent experiments. Differential protein expression of BC-adapted planktonic and biofilm *Salmonella* serovar Enteritidis cells was determined from averaged spot volumes from four experimentally-replicated 2D-PAGE gels. Experimental data were analyzed using SAS statistical software (version 9.1.3; SAS Institute Inc., Cary, NC), and the Fisher's Least Significant Difference (LSD) method was used to test for significant ($P < 0.05$) differences.

6.4. Results

6.4.1. Role of BC adaptation on the survival of planktonic and biofilm cells following lethal BC exposure

Survival of BC-adapted and untreated planktonic and biofilm cells were determined by plating on SPCA containing increasing concentrations of BC. Cells belonging to all four treatments (i.e., BC-adapted and untreated control planktonic and biofilm cells) were unable to grow on SPCA plates at BC concentrations of 35 μg ml^{-1} or more during some of the replications of experiment, thus results from BC concentrations > 30 μg ml^{-1} were eliminated from further analyses (Figure 6.4.1; Tables 6.4.1 and 6.4.2). The number of BC-adapted and untreated planktonic and biofilm cells able to survive on SPCA plates were inversely proportional to the concentration of BC. The most substantial difference in the number of survivors was found at the BC concentration of 30 μg ml^{-1}. There was no significant difference ($P > 0.05$) in the proportion of control (untreated) planktonic (7.8 X 10^3 cfu ml^{-1} at 30 μg ml^{-1}) and biofilm (6.0 X 10^3 cfu ml^{-1} at 30 μg ml^{-1}) cells in their ability to survive in increasing concentrations of BC up to 30 μg ml^{-1}. However, the proportion of BC-adapted biofilm cells (1.1 X 10^5 cfu ml^{-1}) able to survive increasing concentrations of BC up to 30 μg ml^{-1}, was significantly higher ($P < 0.05$) (4.6-fold) than that of BC-adapted planktonic cells (2.4 X 10^4 cfu ml^{-1}). Further, the proportion of survivors among the BC-adapted biofilm cells (18.2-fold) was significantly higher ($P < 0.05$) than the proportion of survivors among BC-adapted planktonic cells (3-fold) at 30 μg ml^{-1} of BC, relative to their respective untreated counterparts. There was complete lethality of the cells from each of the four treatment within 2 min, following the exposure to a lethal (500 μg ml^{-1}) BC concentration. No survivors were observed following further extended exposures (i.e., 4, 6, 8, and 10 min) to 500 μg ml^{-1} BC, indicating that the lethal effect of BC exposure was similar and also significant on all the four treatments (data not shown).

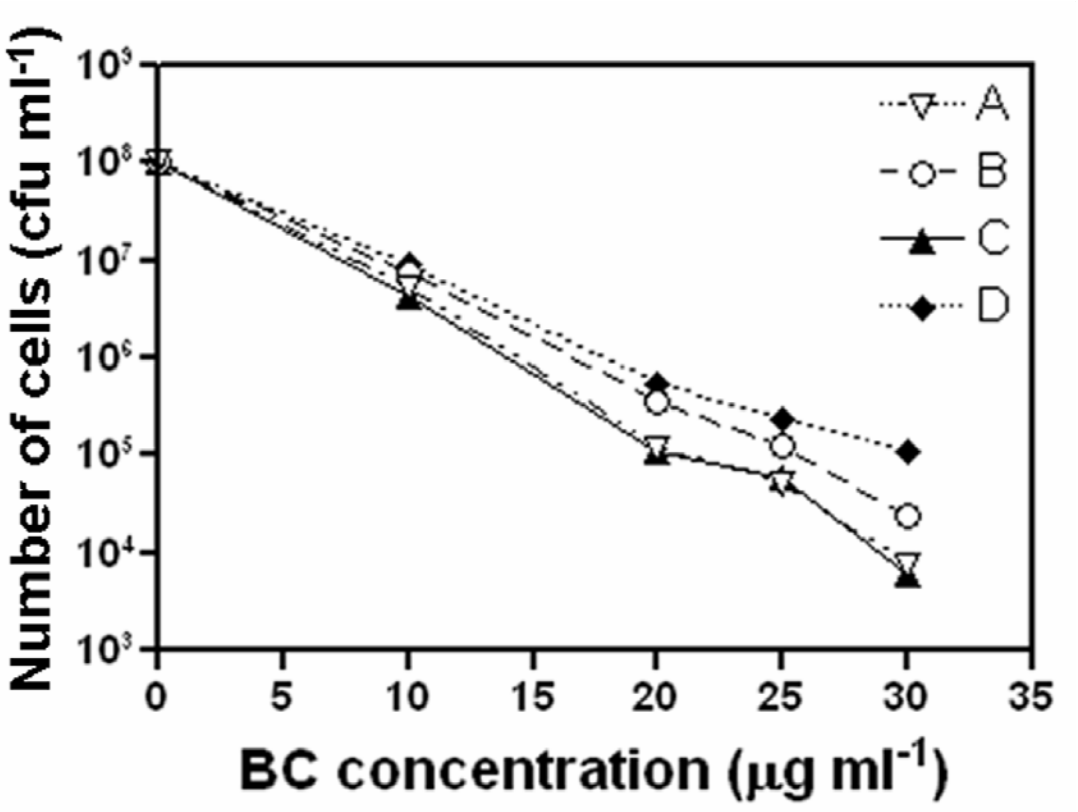


Figure 6.4.1. Survival assay based on plating of *Salmonella* serovar Enteritidis planktonic and biofilm cells on SPCA containing increasing concentrations (0, 10, 20, 25, and 30 μg ml^{-1}) of BC (n = 4). Planktonic cells grown as continuous culture and untreated with BC (planktonic control) (A); planktonic cells grown as continuous culture and adapted to BC (by continuous exposure to 1 μg ml^{-1} of BC for 144 h) (B); biofilm cells untreated with BC (biofilm control) (C); and biofilm cells adapted to BC (by continuous exposure to 1 μg ml^{-1} of BC for 144 h) (D).

Table 6.4.1. Number of survivors of *Salmonella* serovar Enteritidis planktonic and biofilm cells plated on SPCA with increasing concentrations of BC

Concentration of BC ($\mu g\ ml^{-1}$)	Planktonic phenotype		Biofilm phenotype	
	Untreated with BC[a] ($cfu\ ml^{-1}$)	Adapted to BC ($cfu\ ml^{-1}$)	Untreated with BC[a] ($cfu\ ml^{-1}$)	Adapted to BC ($cfu\ ml^{-1}$)
0	1.5×10^8	1.5×10^8	1.5×10^8	1.5×10^8
10	5.3×10^6	7.5×10^6	4.3×10^6	9.0×10^6
20	1.2×10^5	3.6×10^5	1.1×10^5	5.5×10^5
25	5.1×10^4	1.2×10^5	6.1×10^4	2.3×10^5
30	7.8×10^3	2.4×10^4	6.0×10^3	1.1×10^5

[a] Control for respective phenotype.

Table 6.4.2. Proportion of surviving population of *Salmonella* serovar Enteritidis planktonic and biofilm cells on SPCA with increasing concentrations of BC

Concentration of BC ($\mu g\ ml^{-1}$)	Planktonic phenotype		Biofilm phenotype	
	Untreated with BC[a] (%)	Adapted to BC (%)	Untreated with BC[a] (%)	Adapted to BC (%)
0	100.00	100.00	100.00	100.00
10	5.30	7.50	4.30	9.00
20	0.12	0.36	0.11	0.55
25	0.05	0.12	0.06	0.23
30	0.008	0.024	0.006	0.109

[a] Control for respective phenotype.

6.4.2. Survival of heat-shocked BC-adapted and untreated planktonic and biofilm cells

The population of *Salmonella* serovar Enteritidis cells belonging to the four treatments (BC-adapted and untreated planktonic and biofilm cells) able to survive a 10 min heat-shock at 55^{o}C was analyzed (Figure 6.4.2). There was no significant difference ($P > 0.05$) in the survival of untreated and BC-adapted planktonic cell population at different time intervals, with the exception of the 2 and 6 min time intervals (Figure 6.4.2A). The number of surviving BC-adapted biofilm cells was significantly higher ($P < 0.05$) than untreated biofilm cells at most time intervals (i.e., 2, 4, 6, and 10 min) of incubation (Figure 6.4.2B). Overall, the proportion of biofilm cells (BC-adapted and untreated) that survived heat-shock at 55^{o}C was significantly higher than the proportion of planktonic cells (BC-adapted and untreated), especially at 2, 4, 6, 8 and 10 min time intervals. The results obtained for heat-shocked BC-adapted and untreated planktonic and biofilm cells at 62.8^{o}C for 10 min showed that there was complete lethality of the cells belonging to all four treatment groups even within 2 min of incubation (data not shown).

6.4.3. Cell surface roughness changes of BC-adapted and untreated planktonic and biofilm cells

Alterations in the cell surface roughness of BC-adapted and untreated planktonic and biofilm cells were analyzed by AFM and image analyses (Figures 6.4.3 and 6.4.4). The average cell roughness measurements of BC-adapted and untreated planktonic cells and BC-adapted and untreated biofilm cells before exposing to lethal BC concentrations, were 114 ± 37 (average ± standard deviation), 128 ± 44, 135 ± 57, and 215 ± 104 Å, respectively. However, following the exposure to lethal BC concentrations of 30, 50, 100, and 500 μg ml^{-1} for 10 min, the surface roughness of untreated planktonic cells was 92 ± 40, 85 ± 32, 117 ± 49, and 142 ± 48 Å, respectively, and for BC-adapted planktonic cells the surface roughness was 155 ± 49, 202 ± 81, 177 ± 65, and 176 ± 80 Å,

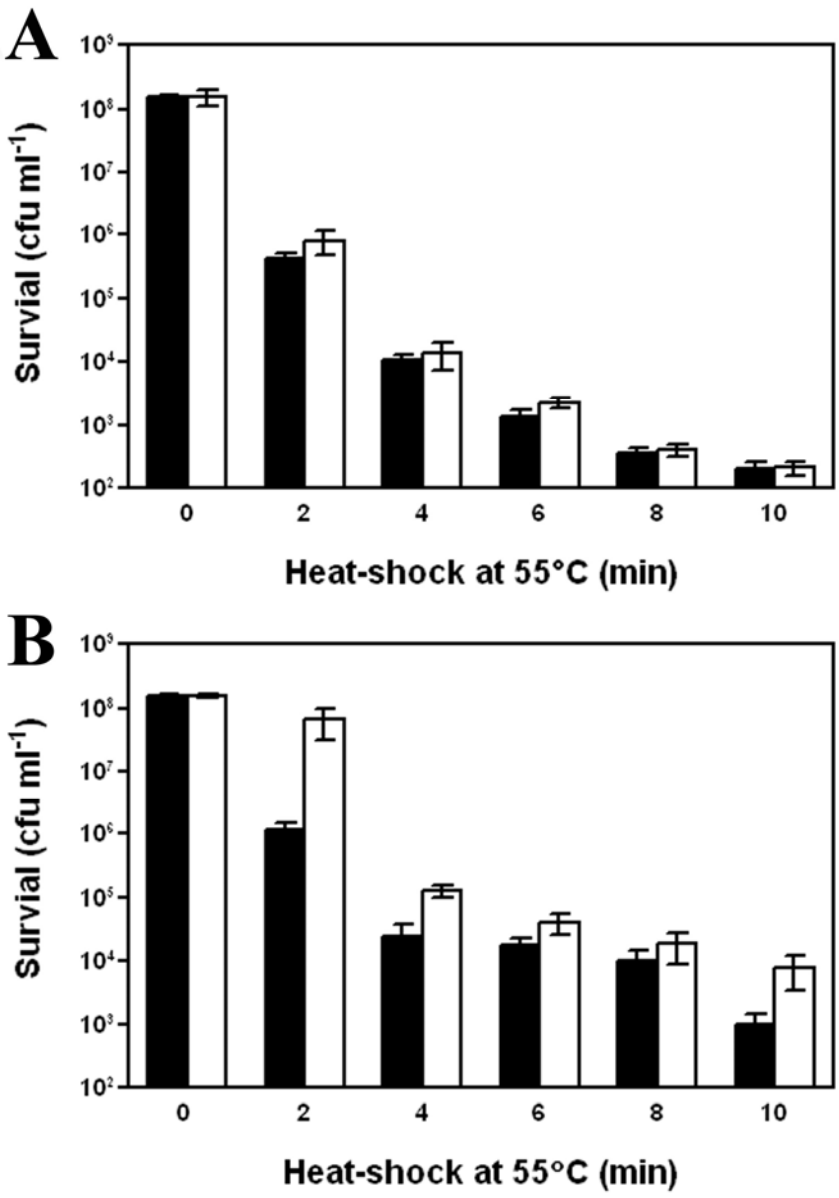


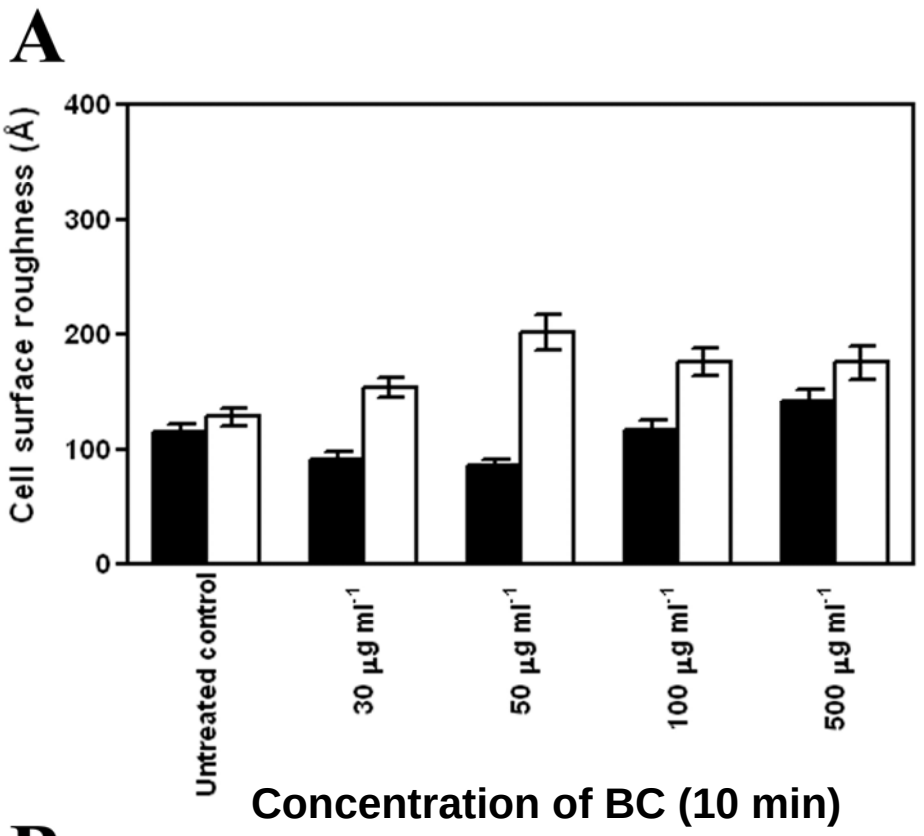
Figure 6.4.2. Survival of untreated and BC-adapted *Salmonella* serovar Enteritidis (A) planktonic and (B) biofilm cells following heat-shock at 55°C for a period of 10 min (n = 4). BC-adaptation of the cells was carried out by continuous exposure to 1 µg ml^{-1} of BC for 144 h. The measurements pertaining to untreated and BC-adapted (planktonic and biofilm) cells are indicated by the symbols (■) and (▭), respectively. The cells were incubated at 55°C for 10 min; samples were collected at every 2 min during the incubation and plated on SPCA plates after serial dilution. Plates were incubated at 37°C for 24 h and the colonies were counted in order to estimate the number of survivors in cfu ml^{-1}. The error bars indicate the standard error of the mean.

respectively. The surface roughness of untreated biofilm cells was 215 ± 88, 259 ± 116, 318 ± 135, and 339 ± 176 Å, respectively, and for BC-adapted biofilm cells surface roughness was 265 ± 110, 242 ± 91, 179 ± 61, and 123 ± 55 Å, respectively. The cell surface roughness of untreated planktonic cells sequentially reduced until exposed to lethal BC concentration of 50 μg ml^{-1}, and then increased with the exposure to further increased lethal BC concentrations. With regard to BC-adapted planktonic cells, the cell surface roughness initially increased while being treated with lethlal BC concentration upto 50 μg ml^{-1} and subsequently reduced on exposure to further increased lethal BC concentrations. In the case of untreated biofilm cells, cell surface roughness increased with the increase in lethal BC concentration. However with respect to BC-adapted biofilm cells, cell surface roughness increased until being treated with 30 μg ml^{-1} of BC, and then reduced with the increase in lethal BC concentration. Overall, the cell surface roughness of biofilm cells was significantly higher ($P < 0.05$) than that of planktonic cells, in the case of both BC-adapted and untreated cells (Figure 6.4.3). Thus, it was deduced that the cell surface roughness was significantly influenced by the phenotype of the cells (i.e., planktonic or biofilm).

6.4.4. Fatty acid profiles of BC-adapted and untreated planktonic and biofilm cells

Fatty acid profiles of BC-adapted and untreated planktonic and biofilm cells were determined by FAME analyses (Table 6.4.3), revealing qualitative and quantitative differences between their fatty acid composition. The profiles consisted primarily of saturated fatty acids (SFA), branched chain fatty acids (BCFA), unsaturated fatty acids (UFA), cyclic fatty acids (CFA), and fatty alcohols (FA). There were also three summed features (i.e., SF 2, 3, and 5), which represent structurally similar fatty acids that could not be resolved by the method used. The profile of untreated planktonic cells was composed of SFA (12:0, 14:0, and 16:0), BCFA (17:1 anteiso/iso and 19:0 anteiso), UFA (18:1 ω7c), CFA (17:0 cyc), as well as SF 2 (14:0 3OH/16:1 iso) and SF 3 (15:0 iso 2OH/16:1 ω7c). The major differences in the FAME profile of BC-adapted planktonic cells from the control cells were the absence of BCFA (17:1 anteiso/iso and 19:0 anteiso) and CFA (17:0 cyc); however, there was also the significantly enhanced ($P < 0.05$) quantity of SFA (12:0) and SF 3. The fatty acid profile of untreated biofilm cells

Figure 6.4.3. (facing page) Average cell surface roughness measurements expressed in Å (n = 60), of *Salmonella* serovar Enteritidis (A) planktonic and (A) biofilm cells, as determined by AFM image acquisition (Figure 6.4.4) and image analyses using PicoScan^{TM} software version 5.3.1a (Molecular Imaging Corp., Tempe, AZ). BC-adaptation of the cells was carried out by continuous exposure to 1 µg ml^{-1} of BC for 144 h. The measurements pertaining to untreated and BC-adapted (planktonic and biofilm) cells are indicated by the symbols (▬) and (▭), respectively. The cells were exposed to increasing lethal concentrations (30, 50, 100, and 500 µg ml^{-1}) of BC for 10 min and then analyzed using AFM. Untreated cells of the respective phenotype were analyzed to serve as controls. The error bars indicate the standard error of the mean.


A
Cell surface roughness (Å)
400
300
200
100
0
Untreated control
30 µg ml-1
50 µg ml-1
100 µg ml-1
500 µg ml-1
Concentration of BC (10 min)

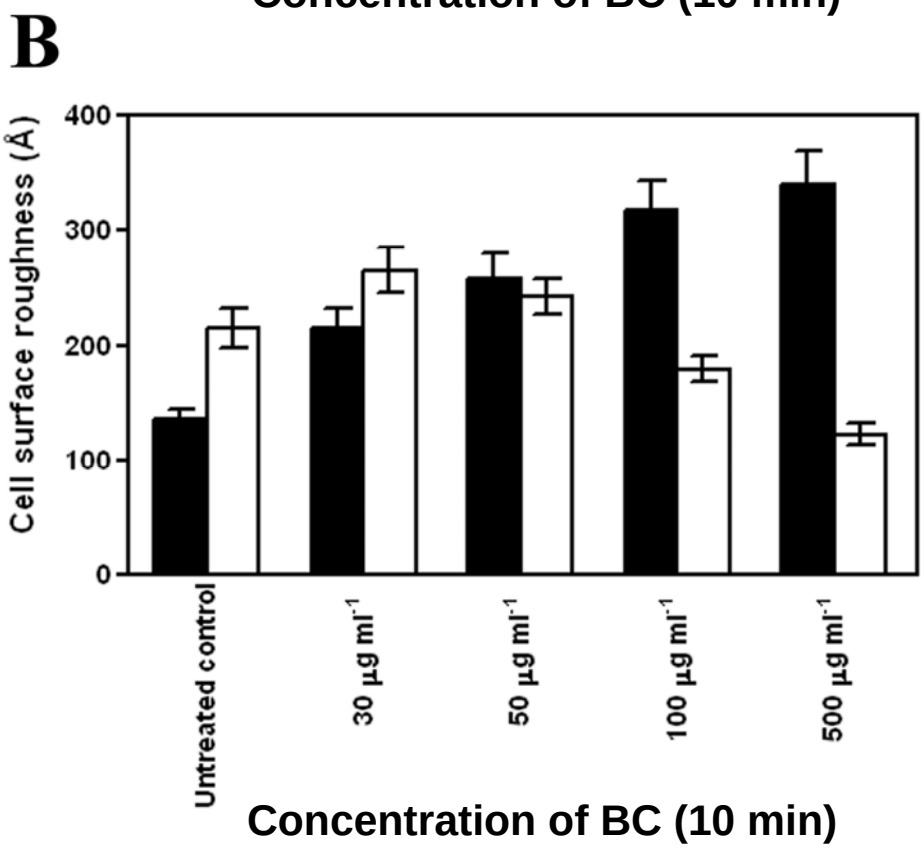
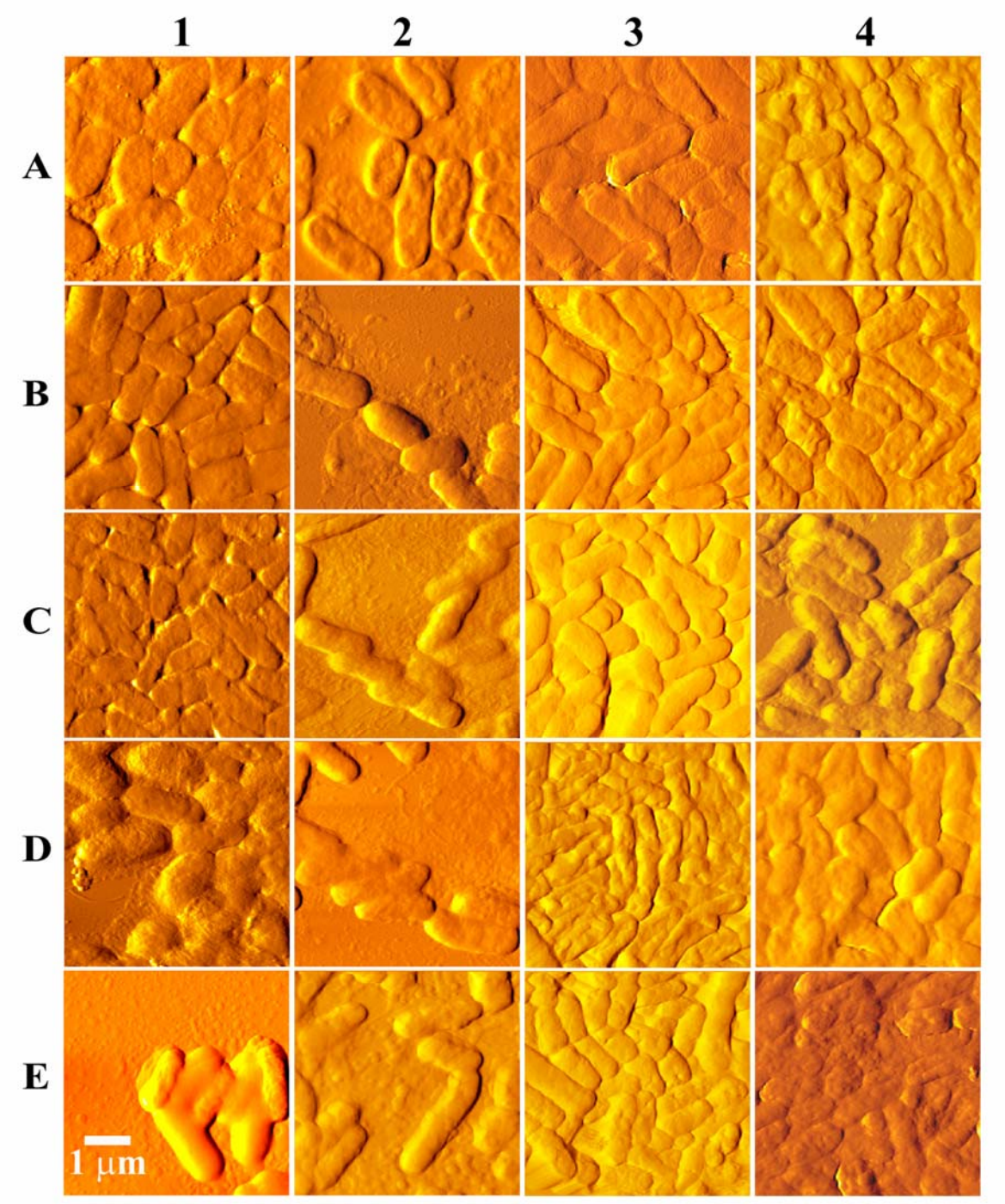

B
Cell surface roughness (Å)
400
300
200
100
0
Untreated control
30 µg ml-1
50 µg ml-1
100 µg ml-1
500 µg ml-1
Concentration of BC (10 min)

Figure 6.4.4. (facing page) Representative AFM images showing the effect of lethal BC treatment (30, 50, 100, and 500 μg ml^{-1} of BC for 10 min) on untreated and BC-adapted planktonic and biofilm cells of *Salmonella* serovar Enteritidis. The images were analyzed using PicoScan™ software version 5.3.1a (Molecular Imaging Corp., Tempe, AZ) to determine the cell surface roughness changes in Å (n = 60) following the BC treatments (Figure 6.4.3). The columns (1 to 4) represent the images pertaining to untreated planktonic cells, BC-adapted planktonic cells, untreated biofilm cells, and BC-adapted biofilm cells, respectively. The row (A) represents the cells which were not exposed to the lethal BC treatments. The rows (B to E) represent the cells treated with increasing lethal BC concentrations of 30, 50, 100, and 500 μg ml^{-1} for 10 min, respectively.


1
2
3
4
A
B
C
D
E
1 μm

consisted of SFA (12:0, 14:0, 15:0, 16:0, and 17:0), BCFA (15:1 anteiso), UFA (17:1 ω9c and 18:1 ω7c), CFA (17:0 cyc and 19:0 cyc), as well as SF 2 and SF 3. There was also a significant difference in the composition of fatty acids between untreated and BC-adapted biofilm cells, in terms of number of fatty acids changed and their relative proportion, including the absence of SFA (17:0), BCFA (15:1 anteiso), UFA (17:1 ω9c), and CFA (19:0 cyc), as well as the significant reduction ($P < 0.05$) in the proportion of SFA (12:0, 14:0, 15:0, and 16:0), UFA (18:1 ω7c), CFA (17:0 cyc), as well as SF 2 and SF 3 in BC-adapted biofilm cells. However, there was production of BCFA (16:0 anteiso), UFA (14:1 ω7c, 15:1 ω7c, and 16:1 ω7c), FA (16:1 ω7c alcohol), and SF 5 (18:0 anteiso/18:2 ω6, 9c) in BC-adapted biofilm cells (Table 6.4.3). There were also significant differences in the fatty acid composition between untreated planktonic and biofilm cells and between BC-adapted planktonic and biofilm cells. With respect to the untreated control cells, BCFA (17:1 anteiso/iso and 19:0 anteiso) and significantly enhanced ($P < 0.05$) biosynthesis of SFA (12:0) was seen in planktonic cells. However, the FAME profile of untreated biofilm cells included SFA (15:0 and 17:0), BCFA (15:1 anteiso), UFA (17:1 ω9c), and CFA (19:0 cyc), as well as significantly enhanced ($P < 0.05$) biosynthesis of CFA (17:0 cyc) relative to untreated planktonic cells. With regard to BC adaptation, there was significantly enhanced ($P < 0.05$) biosynthesis of SFA (12:0, 14:0, and 16:0), UFA (18:1 ω7c), as well as SF 2 and SF 3 in BC-adapted planktonic cells. However, there was production of SFA (15:0), BCFA (16:0 anteiso), UFA (14:1 ω5c, 15:1 ω5c, and 16:1 ω5c), CFA (17:0 cyc), FA (16:1 ω7c alcohol), and SF 5 in BC-adapted biofilm cells (Table 6.4.3).

6.4.5. Proteomic analyses of BC-adapted and untreated planktonic and biofilm cells

There were 40 differentially-expressed proteins following *Salmonella* serovar Enteritidis biofilm adaptation to BC (Mangalappalli-Illathu and Korber, 2006). Five proteins were up-regulated (Figure 6.4.5; Table 6.4.4) and seven proteins were down-regulated (Figure 6.4.5; Table 6.4.5) in both planktonic and biofilm cells following BC adaptation. Moreover, 39 differentially-expressed proteins were seen in both planktonic and biofilm cells following BC adaptation (Table 6.4.6). The up-regulated proteins were

Table 6.4.3. Fatty acid composition of *Salmonella* serovar Enteritidis planktonic and biofilm cells following adaptation to BC*

Fatty acids	Planktonic phenotype		Biofilm phenotype	
	Untreated with BC[a] (% ± SD)[b]	Adapted to BC (% ± SD)[b]	Untreated with BC[a] (% ± SD)[b]	Adapted to BC (% ± SD)[b]
Saturated fatty acids				
12:0	4.0 ± 0.3	4.6 ± 0.2	2.7 ± 0.1	2.0 ± 0.0
14:0	8.3 ± 2.3	8.1 ± 0.4	7.1 ± 1.4	2.9 ± 0.3
15:0	ND[d]	ND	1.0 ± 0.1	0.7 ± 0.0
16:0	22.0 ± 1.9	24.3 ± 1.9	23.7 ± 2.7	9.7 ± 0.6
17:0	ND	ND	1.7 ± 2.1	ND
Branched chain fatty acids				
15:1 anteiso	ND	ND	0.7 ± 0.0	ND
16:0 anteiso	ND	ND	ND	17.9 ± 0.7
17:1 anteiso/iso	7.4 ± 0.0	ND	ND	ND
19:0 anteiso	3.8 ± 0.0	ND	ND	ND
Unsaturated fatty acids				
14:1 ω5c	ND	ND	ND	5.5 ± 1.0
15:1 ω5c	ND	ND	ND	0.7 ± 0.0
16:1 ω5c	ND	ND	ND	19.4 ± 0.8
17:1 ω9c	ND	ND	8.3 ± 0.0	ND
18:1 ω7c	18.7 ± 2.2	20.1 ± 2.5	18.0 ± 3.4	7.4 ± 1.2

Continued…

Table 6.4.3. continued.

Fatty acids	Planktonic phenotype		Biofilm phenotype	
	Untreated with BC[a] (% ± SD)[b]	Adapted to BC (% ± SD)[b]	Untreated with BC[a] (% ± SD)[b]	Adapted to BC (% ± SD)[b]
Cyclic fatty acids				
17:0 cyc	2.9 ± 0.1	ND	4.8 ± 0.5	0.9 ± 0.0
19:0 cyc	ND	ND	0.7 ± 0.0	ND
Fatty alcohols				
16:1 ω7c alcohol	ND	ND	ND	4.9 ± 0.0
Summed feature[c]				
2 (14:0 3OH/16:1 iso)	7.5 ± 0.1	8.1 ± 0.5	7.1 ± 1.4	5.0 ± 0.5
3 (15:0 iso 2OH/16:1 ω7c)	25.3 ± 3.5	34.8 ± 3.5	24.2 ± 4.7	11.2 ± 0.9
5 (18:0 anteiso/18:2 ω6, 9c)	ND	ND	ND	11.9 ± 1.9

* Data from each treatment were the mean from at least three independent replications.
[a] Control for corresponding phenotype.
[b] Proportion out of total cellular fatty acid methyl ester (FAME) (100%) ± standard deviation.
[c] Denotes structurally similar fatty acids that cannot be resolved by the method employed.
[d] ND, Not detected.

TrxA (biosynthesis), Tsf (protein translation and modification), CspA (adaptation), YjgF (a hypothetical protein), and a probable peroxidase STY0440 (putative thiol-alkyl hydroperoxide reductase) (detoxification); the down-regulated proteins were GarR (degradation), GpmA (glycolysis), RpsA (ribosomal protein synthesis and modification), TufA (protein translation and modification), RfbH (cell envelope synthesis), OppA (oligopeptide-binding protein), and DnaK (chaperone protein Hsp70). Nine unique proteins were up-regulated in BC-adapted planktonic cells and 17 unique proteins were up-regulated in BC-adapted biofilm cells. It was further observed that following BC adaptation, the majority of proteins that were up-regulated in biofilm cells were either unchanged in expression or down-regulated in planktonic cell proteome, and *vice versa* (Table 6.4.6).

6.5. Discussion

The ability of BC-adapted and untreated planktonic and biofilm cell populations to survive increasing lethal BC concentrations and heat-shock was examined by following cell surface roughness, cellular fatty acid composition, and protein differential expression. Comparable studies have been reported for different bacterial species against various antimicrobial agents (Joseph et al., 2001; Spoering and Lewis, 2001; Scher et al., 2005). Generally, it has been found that biofilm cells are more tolerant to antimicrobial agents than their planktonic counterparts. The survival of untreated (control) planktonic and biofilm cells was similar during exposure to increasingly lethal BC concentrations, especially up to 30 μg ml^{-1} concentration (in solid or agar medium). However, there was a significant increase ($P < 0.05$) in the ability of BC-adapted planktonic and biofilm cell populations able to survive in the same increasing BC concentrations over their untreated counterparts. Moreover, the proportion of BC-adapted biofilm cells able to survive lethal BC concentrations (up to 30 μg ml^{-1}) was significantly higher ($P < 0.05$) than for the BC-adapted planktonic cells. In contrast, there was complete lethality of the controls, as well as BC-adapted, planktonic and biofilm cells exposed to 500 μg ml^{-1} of BC (in liquid or broth medium) for 10 min. Enhanced survival of bacteria when exposed to low lethal BC concentration (in solid medium) was significantly influenced by previous exposure to BC and concurrent with changes in the cell phenotype. Similar to

Figure 6.4.5. (facing page) Total proteins of p*I* values ranging from 4 and 7, which were differentially-expressed in both planktonic and biofilm cells of *Salmonella* serovar Enteritidis adapted to BC. The images illustrate the representative 2D-PAGE gels pertaining to proteins extracted from untreated control planktonic cells (A), planktonic cells adapted to continuous exposure to BC (1 μg ml^{-1}) (B), untreated control biofilm cells (C), and biofilm cells adapted to continuous exposure to BC (1 μg ml^{-1}) (D). The description of the proteins and their levels of expression are illustrated in Table 6.4.4 (up-regulation) and Table 6.4.5 (down-regulation). The symbol (□) indicate the location of the protein spot in the controls; the symbol (△) indicates up-regulation, and (▽) indicates down-regulation of the protein relative to the expression in the corresponding untreated controls.

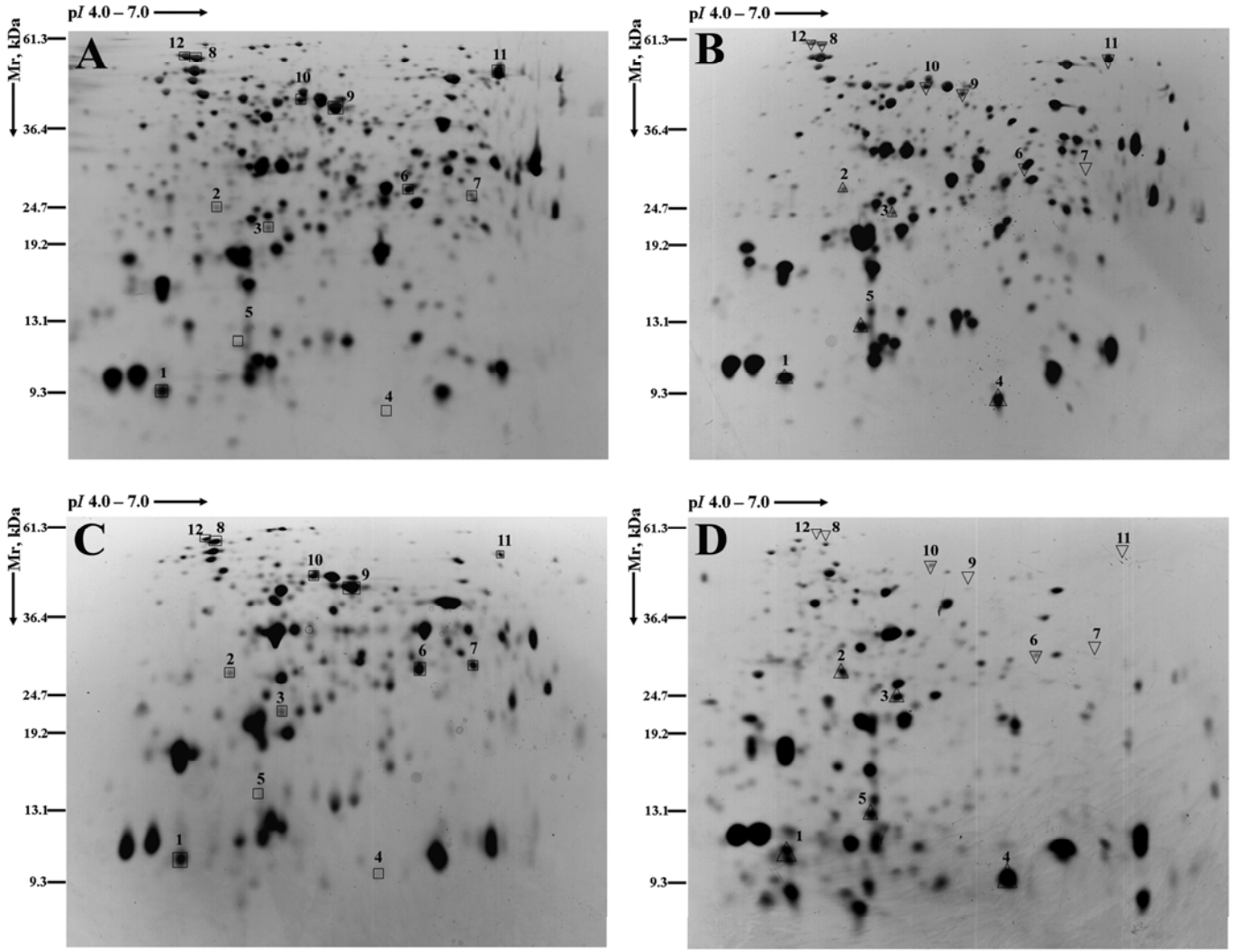

A
B
C
D
pI 4.0 – 7.0
Mr, kDa
61.3
36.4
24.7
19.2
13.1
9.3
1
2
3
4
5
6
7
8
9
10
11
12

Table 6.4.4. Proteins up-regulated in planktonic and biofilm cells of *Salmonella* serovar Enteritidis following continuous sub-lethal (1 μg ml^{-1}) BC exposure for 144 h

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold increase[b]	
						Planktonic cells	Biofilm cells
Biosynthesis of cofactors, prosthetic groups, and carriers	1	*trxA*	Thioredoxin 1	11.68	4.67	1.58	1.86
Protein translation and modification	2	*tsf*	Protein chain elongation factor (EF-Ts)	30.36	5.13	1.54	8.81
Cell processes (detoxification)	3	NK[c]	Probable peroxidase STY0440	22.32	5.24	3.88	6.75
Adaptation and atypical conditions	4	*cspA*	7.4-kDa cold-shock protein	7.27	5.57	32.10	110.13
Hypothetical proteins	5	*yjgF*	Conserved hypothetical protein YjgF	13.57	5.36	6.84	33.81

Mr, molecular mass; p*I*, isoelectric point.

[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.

[b] Fold increase in protein expression from that of BC-untreated planktonic and biofilm cells, respectively.

[c] NK, Not known.

Table 6.4.5. Proteins down-regulated in planktonic and biofilm cells of *Salmonella* serovar Enteritidis following continuous sub-lethal (1 μg ml^{-1}) BC exposure for 144 h

Class and function	Spot no.	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold decrease[b]	
						Planktonic cells	Biofilm cells
Degradation (carbon compounds)	6	*garR*	Tartronate semialdehyde reductase	30.73	5.59	0.58	0.09
Energy metabolism (glycolysis)	7	*gpmA*	Phosphoglycerate mutase 1	28.36	5.78	0.46	0.02
Ribosomal protein synthesis and modification	8	*rpsA*	30S ribosomal subunit protein S1	61.25	4.89	0.38	0.08
Protein translation and modification	9	*tufA*	Translation elongation factor (EF-Tu.A)	43.40	5.30	0.27	0.25
Cell envelope (lipopolysaccharides)	10	*rfbH*	Lipopolysaccharide biosynthesis protein	48.10	5.27	0.66	0.06
Cell processes (binding proteins [other])	11	*oppA*	Oligopeptide-binding protein complexed with Kvk, chain A	58.81	5.85	0.54	0.09
Cell processes (chaperones)	12	*dnaK*	Chaperone protein Hsp70	69.13	4.83	0.65	0.17

Mr, molecular mass; p*I*, isoelectric point.

[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.

[b] Fold decrease in protein expression from that of BC-untreated planktonic and biofilm cells, respectively.

Table 6.4.6. Proteins that differentially-expressed in planktonic and biofilm cells of *Salmonella* serovar Enteritidis following continuous sub-lethal (1 μg ml^{-1}) BC exposure for 144 h

Class and function	Gene	Protein description	Mr[a] (kDa)	pI[a]	Fold change[b]	
					Planktonic cells	Biofilm cells
Degradation (carbon compounds)	*pduA*	Putative propanediol utilization protein A	9.59	6.72	0.79*	5.25
	pduJ	Putative propanediol utilization protein J	9.07	6.50	1.00*	84.76
Degradation (proteins, peptides, and glycoproteins)	*degQ*	Serine endoprotease	47.28	6.80	0.92*	0.28
Energy metabolism (ATP synthesis)	*atpA*	ATP synthase *α*-subunit	54.98	5.80	0.77*	0.13
Energy metabolism (glycolysis)	*eno*	Enolase	45.47	5.25	0.32	36.54
	fbaA	Fructose 1,6-bisphosphate aldolase class II	39.36	5.58	0.96*	0.18
	gapA	Glyceraldehyde-3-phosphate dehydrogenase A	35.46	6.32	0.79*	0.11
	pgk	Phosphoglycerate kinase	41.00	5.09	0.19	1.12*
	tpiA	Triose phosphate isomerase	26.92	5.68	0.98*	3.27
Energy metabolism (tricarboxylic acid cycle)	*mdh*	Malate dehydrogenase	32.48	6.01	0.73*	0.02
Energy metabolism (non-oxidative phase of pentose phosphate pathway)	*rpiA*	Ribose-5-phosphate isomerase A	22.90	5.08	0.97*	0.42
	talB	Transaldolase B	35.04	5.09	5.99	0.83*
Energy metabolism (glyoxylate bypass)	*aceA*	Isocitrate lyase	47.56	5.22	2.15	0.78*
	aceK	Isocitrate dehydrogenase kinase/phosphatase	46.09	5.99	0.87*	0.48

Continued…

Table 6.4.6. continued.

Class and function	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold change[b]	
					Planktonic cells	Biofilm cells
Aromatic amino acid biosynthesis	*wrbA*	Flavoprotein WrbA	20.74	5.79	0.42	8.10
Ribosomal protein synthesis and modification	*rplL*	50S ribosomal subunit proteins L7 and L12	12.17	4.60	1.00*	1.78
	rplL	50S ribosomal subunit proteins L7 and L12	12.17	4.60	0.89*	1.87
	rpsB	30S ribosomal subunit protein S2	26.76	6.62	5.60	0.70*
Protein translation and modification	*tuf*	Elongation factor Tu (fragment)	29.29	5.30	0.81*	41.83
	dsbA	Thiol:disulfide interchange protein	22.91	5.64	1.19*	2.48
RNA synthesis, RNA modification, and DNA transcription	*rpoZ*	DNA-directed RNA polymerase ω-chain	10.24	4.87	0.55	4.61
Cell envelope (surface structures)	*fljB*	Phase 2 flagellin	52.41	4.75	0.33	1.02*
	fliC	Phase 1 flagellin	51.48	4.79	0.30	1.23*
Cell processes (amino acid-binding proteins)	*argT*	Lysine-arginine-ornithine-binding periplasmic protein	28.20	5.99	2.52	0.89*
	artI	Arginine-binding periplasmic protein 1 precursor	27.00	7.66	1.73	0.24
Cell processes (carbohydrate-binding proteins)	*crr*	Phosphotransferase system enzyme II (glucose-permease IIA component)	18.12	4.73	4.17	1.08*
	fruB	Phosphotransferase system enzyme II (fructose-specific IIA/FPr component)	39.59	4.87	0.71*	4.17
	malE	Maltose-binding periplasmic protein	43.18	6.27	0.66	0.76*
	mglB	D-Galactose/D-glucose-binding periplasmic protein	35.81	5.81	0.93*	0.57

Continued…

Table 6.4.6. continued.

Class and function	Gene	Protein description	Mr[a] (kDa)	p*I*[a]	Fold change[b]	
					Planktonic cells	Biofilm cells
Cell processes (binding proteins [other])	*ptsH*	Phosphocarrier protein HPr (histidine-containing protein)	9.12	5.65	1.50	1.41*
Cell processes (detoxification)	*sodB*	Superoxide dismutase [Fe]	21.18	5.58	0.34	27.13
	tpx	Thiol peroxidase	17.98	4.75	1.42*	36.82
Broad regulatory functions	*hns*	DNA-binding protein H-NS (histone-like protein II)	15.41	5.32	1.85	0.11
Atypical conditions and adaptation	*grcA*	Autonomous glycyl radical cofactor	14.34	5.10	2.24	1.03*
	uspA	Universal stress protein A	15.95	5.12	0.88*	0.18
Hypothetical proteins	*gntY*	Hypothetical protein GntY	20.94	4.52	1.14*	30.70
	ycbL	Hypothetical protein YcbL (putative metallo-*β*-lactamase)	23.73	4.95	0.31	6.27
	ynaF	Conserved hypothetical protein STY1416 (putative universal stress protein)	15.70	5.93	1.13*	2.06
Miscellaneous proteins	NK[c]	Putative periplasmic protein (*Salmonella* serovar Typhi strain CT18)	21.44	NK[c]	0.98*	2.86

Mr, molecular mass; p*I*, isoelectric point.

[a] Theoretical values obtained from Swiss-Prot or PUMA2 databases.

[b] Fold change in protein expression from that of BC-untreated planktonic and biofilm cells, respectively.

[c] NK, Not known.

[*] Unchanged level of expression (i.e., 0.67 – 1.49).

the present results, *P. aeruginosa* adapted to BC by serial passage showed an increased resistance (i.e., MIC), from 0.003 to 0.05 (%w/v) (Loughlin et al., 2002). Moreover, phenotypic changes such as alterations in outer membrane proteins and fatty acid composition, cell surface charge, as well as hydrophobicity were associated with the BC-resistance of *P. aeruginosa*. However, Langsrud et al. (2004) demonstrated that BC resistance of *E. coli* could be increased to 150 μg ml^{-1} or more by serial passage of bacteria in higher BC concentrations daily for longer period (25 to 27 days). It is therefore likely that the MIC of *Salmonella* serovar Enteritidis ATCC 4931 to BC could have been increased to ~150 μg ml^{-1} by simply extending the adaptation period.

The survival of cells to heat-shock at 55°C was also significantly influenced ($P < 0.05$) by both the biofilm phenotype and BC treatment. The number of surviving control biofilm cells was significantly higher ($P < 0.05$) than the control planktonic cells following heat treatment at 55°C for 10 min. Moreover, the BC-adapted biofilm cells were better able to survive than the control biofilm cells. However, there was no significant difference ($P > 0.05$) between the survival of BC-adapted and untreated control planktonic cell populations when exposed to the same heat-shock challenge. Thus, the survival of heat-shocked cells (55°C) was influenced by both the biofilm-phenotype and BC adaptation. Notably, the same results were not seen following heat-shock at 62.8°C, suggesting that the temperature (i.e., a pasteurization temperature) is lethal and that the organism was unable to get any protection because of the phenotype and BC adaptation. Similar to the present findings, Sampathkumar et al. (2004) reported that sub-lethal trisodium phosphate (TSP) treatment induced thermotolerance in *Salmonella* serovar Enteritidis ATCC 4931, and suggested that *de novo* protein synthesis and altered membrane fatty acid composition were possible mechanisms.

The possibility that phenotypic shifting (from biofilm to planktonic) occurred during these experiments was minimized by the quick processing/treating of the cells and immediate plating. However, there exists the possibility that some transformation of the cells from biofilm- to planktonic-phenotype following biofilm disruption for sample preparation occurred. If this were the case, there should actually be more survivors among the BC-adapted biofilm cell population than was observed in the survival assay and heat-shock. With regard to survival of heat-shock (55°C), the control biofilm cells

were better survivors than the control planktonic cells; whereas, the survival of control planktonic and biofilm cells were more or less similar in increasing lethal BC concentrations (up to 30 μg ml^{-1}). This is similar to the findings that the two phenotypes responded to various stressors (such as physical (heat) and chemical (hypochlorites) treatments) in distinct manner depending on the phase of growth of the cells, as reported previously (Scher et al., 2005). Moreover, Mikkelsen et al. (2007) recently reported that the biofilm cell proteome more closely resembled exponentially-growing planktonic cells than planktonic cells in the stationary phase. Thus, the phase of cell growth and the nature of stressor are key factors that interplay with cell phenotype to determine the outcome of a stressor's action on bacterial cells.

AFM has been employed to characterize surface changes associated with growth phases, mutations, and antimicrobial exposure in bacteria, with considerable resolution (Braga and Ricci, 1998; Cross et al., 2006; Del Sol et al., 2007). The major changes associated with antimicrobial exposure of bacteria are seen on their cell surface. The main target of action of BC is the bacterial cell wall. Following adsorption to the cells, this compound causes cell wall disruption and leakage of cellular constituents based on the concentration (Maxcy et al., 1971). Hoffmann et al. (1973) reported that the surfaces of BC-resistant *P. aeruginosa* cells were covered by an additional layer, which has been described as an unidentified substance, not present on sensitive cells. Sampathkumar et al. (2003) reported that *Salmonella* serovar Enteritidis cells treated with TSP showed disfigured cell surface topology and a wrinkled appearance, along with the disruption of cytoplasmic and outer membranes. Thus, it was presumed that major changes associated with BC adaptation and lethal BC treatment would include alterations in the cell surface roughness mediated through altered surface composition and formation of additional structures, which function to reduce overall cell damage. The average cell surface roughness of untreated biofilm cells was significantly higher ($P < 0.05$) than that of untreated planktonic cells, both before and after exposure to lethal BC concentrations. On exposure to lethal BC concentrations, the surface roughness of untreated planktonic cells initially became reduced (until exposure to 50 μg ml^{-1} of BC) and thereafter increased. However, in the case of untreated biofilm cells, the cell surface became increasingly rough with increasing BC concentration (up to the maximum applied

concentration of 500 μg ml^{-1}). In the case of BC-adapted cells, the average cell surface roughness initially increased, and subsequently became reduced for both planktonic and biofilm cells; however, the change was more pronounced for the BC-adapted biofilm cells. It was found that various aspects such as phenotype, BC adaptation, and lethal BC exposure could significantly alter cell surface roughness of *Salmonella* serovar Enteritidis cells. Similarly, Braga and Ricci (1998) also reported that the damage induced by cefodizime treatment on *E. coli* cells significantly varied depending on the concentration and incubation time of the antibiotic treatment, when the cells were analyzed for fine morphological and surface alterations using AFM. Further, Cross et al. (2006) recently reported that cariogenicity, a virulence trait of *S. mutans*, was inversely proportional to cell surface roughness as determined by AFM. The present experiments are suggestive of an influence of the phenotype (i.e., planktonic or biofilm) on bacterial cell surface roughness, with potential effects on bacterial survival following lethal BC treatments. This is the first report of alterations in bacterial cell surface roughness following sub-lethal and lethal BC treatments. While there was no apparent trend between BC treatment, adaptation, and cell surface roughness, there were interesting effects observed, and it is clear that the cell wall would be a key site of interaction with this compound.

It is presumed that compositional changes (e.g., membrane fatty acids and proteins) in the cell envelope due to phenotype and BC adaptation were involved in the observed altered cell surface roughness responses. Fatty acid profiles of bacterial cells have been found to be significantly influenced by both physical and chemical agents in the microenvironment, and have been suggested as a mechanism for survival and adaptation (Sakagami et al., 1989; Annous et al., 1997; Sampathkumar et al., 2004). For example, BC-resistant *P. aeruginosa* cells were found to contain increased lipid levels (Hoffmann et al., 1973). The acid-shock response induced the formation of cyclopropane fatty acids in *Salmonella* serovar Typhimurium (Kim et al., 2005). In the present study, some major differences in the fatty acid profiles of untreated (control) planktonic and biofilm cells were noticed. Similarly, the shift in fatty acid profiles of planktonic and biofilm cells following BC adaptation was also distinct (Table 6.4.3). The adaptive response to BC in the planktonic phenotype was mediated mainly through SF 3;

whereas, in the biofilm phenotype it was mediated via BCFA (16:0 anteiso), UFA (14:1 ω5c, 15:1 ω5c, and 16:1 ω5c), FA (16:1 ω7c alcohol), and SF 5. This result (i.e., biosynthesis of excess UFA and reduction in CFA content) could be correlated with the report of cold-shock response induced by the biofilm cells of *Salmonella* serovar Enteritidis ATCC 4931 during BC adaptation (Mangalappalli-Illathu and Korber, 2006). There is also a report that TSP treatment of the same organism resulted in a higher saturated and cyclic to unsaturated fatty acid ratio and induced thermotolerance (Sampathkumar et al., 2004). It was suggested that there was significant influence of the phenotype and the BC adaptation on the fatty acid profile of *Salmonella* serovar Enteritidis. The BC adaptation of biofilm cells has resulted in more a complex shift in fatty acid profile, in terms of both number and proportion of fatty acids involved, relative to that of the planktonic cells. Similar to the findings in this study, the significance of accumulation of phospholipids as well as fatty and neutral lipids in the cell walls of BC-resistant *P. aeruginosa* has been reported (Sakagami et al., 1989). Loughlin et al. (2002) reported that membrane fatty acid changes associated with BC adaptation of *P. aeruginosa* included an increase in the proportion of SFA (14:0 and 16:0); whereas, there was a decrease in the proportion of an unknown fatty acid eluting between 2OH 10:0 and 12:0. BCFA (15:0 anteiso) that has been associated with low temperature growth in *L. monocytogenes*, possibly by maintaining a fluid, liquid-crystalline state in the membrane lipids (Annous et al., 1997). The majority of the fatty acids found in this study might have been associated with cell membranes (cell envelope) where they acted as a protective mechanism against the action of BC. Thus, a phenotype-specific shift in membrane fatty acid composition along with the induction of a wide variety of fatty acids is suggested to be a mechanism for enhanced survival of BC-adapted biofilm cells to lethal BC treatment and heat-shock.

The survival of untreated biofilm cells after heat-shock at 55^{o}C indicated that the cell surface alterations (i.e., increase in cell surface roughness or associated changes) of the biofilm-phenotype might have influenced the survival. The ability of cells to survive heat-shock at 55^{o}C was influenced by phenotype because there was a significant difference in the survival of the control planktonic and biofilm cells. However, the other parameter studied (i.e., the ability of cells to survive in increasingly lethal BC

concentration up to 30 μg ml^{-1}) was not influenced by phenotype. It is suggested that phenotype-specific changes in cell surface roughness, fatty acid composition, and protein expression profile following BC adaptation, contributed to the enhanced survival of BC-adapted biofilm cells. The biofilm phenotype by itself could significantly influence survival against lethal agents, or it may also be possible that the phenotype-specific expression triggered by prolonged adaptation phase that confer resistance against these compounds. It has been observed in the current study that once adapted to BC, biofilm cells were more resistant to BC than the planktonic cells. Simões et al. (2005) reported that the biofilm cells of *P. fluorescens* were more resistant to cetyltrimethylammonium bromide (a QAC) relative to their planktonic counterparts. Even though, the phenotype has a significant influence on the survival of bacteria, it may again depend on the type of agent, species of organism, stage of growth cycle etc.

There was no significant difference between the planktonic and biofilm cells exposed to lethal BC concentration of 500 μg ml^{-1}, as complete lethality of both phenotypes was observed within 2 min following BC exposure. Previously, we have determined that the BC-adapted biofilms were capable of significant re-growth following the lethal BC exposure (500 μg ml^{-1} for 10 min) relative to their untreated biofilm counterparts (Mangalappalli-Illathu and Korber, 2006). Thus, the biofilm phenotype-specific enhanced up-regulation of the key proteins (CspA, TrxA, Tsf, YjgF, and a probable peroxidase STY0440 (putative thiol-alkyl hydroperoxide reductase)) coupled with the up-regulation of 17 unique proteins (Table 6.4.6) may have facilitated the BC-adapted biofilm cells to efficiently re-grow following the lethal BC treatment. Poor penetration of BC into the deeper layers of the biofilms and detoxification processes may influence BC adaptation in biofilms. The role of the above proteins in the adaptive resistance of *Salmonella* serovar Enteritidis biofilms to BC has been described previously (Mangalappalli-Illathu and Korber, 2006). There was a significant difference in the expression of two proteins involved in protection against oxidative stress (Tpx and SodB) between BC-adapted planktonic and biofilm cells (Table 6.4.6). Bore et al. (2007) recently reported the up-regulation of proteins involved in protection against oxidative stress (manganese superoxide dismutase (MnSOD)) in BC-adapted *E. coli* cells. In the present study, BC-adapted biofilm cells up-regulated Tpx and SodB, whereas, planktonic

cells did not up-regulate these proteins. There is a recent report that the proteins involved in oxidative stress are highly expressed in natural biofilms (Ram et al., 2005), possibly because of the depletion of locally available nutrients due to increased cell densities. It is suggested that similar differences in the expression of proteins involved in degradation (PduA and PduJ), amino acid and protein biosynthesis (DsbA, RplL, RpoZ, Tuf, and WrbA), and nutrient-binding (FruB) as well as proteins with hypothetical (GntY, YnaF, and YcbL) and unknown functions (putative periplasmic protein) between BC-adapted planktonic and biofilm cells explain the enhanced survival of BC-adapted biofilm cells. The down-regulation of RfbH (LPS biosynthesis protein) in both planktonic and biofilm cells was puzzling. A possible explanation was that the BC concentration (1 μg ml^{-1}) used for the adaptation procedure was low enough to stimulate the biosynthesis of this protein and that the organism may not have sensed an immediate stress, even though the cell envelope is the primary target site of action of this compound. Moreover, other adaptive responses might have been sufficient enough to provide enough resistance. Similar to the observation in this study, Loughlin et al. (2002) reported that there were several phenotypic changes associated with BC adaptation of *P. aeruginosa* cells; however, there was no alteration of the LPS composition. Recently, Braoudaki and Hilton (2005) also reported that there was no significant change in the outer membrane and LPS when *Salmonella* spp. was exposed to sub-lethal BC treatment. Thus, poor penetration of the agent into the deeper layers of biofilms, along with the phenotype-specific adaptive responses had facilitated the deeply-embedded BC-adapted biofilm cells with a better chance to survive and possibly re-grow than biofilm cells that had not been exposed to BC.

Adaptive resistance to antimicrobial agents, including BC, and the cross-resistance of adapted strains to related or unrelated antimicrobial agents have been reported among the members of Family *Enterobacteriaceae*, which comprise many important human pathogens (Braoudaki and Hilton, 2004, 2005; Langsrud et al., 2004). In the present study, there were significant differences in the pattern and degree of resistance of planktonic and biofilm cells to BC as well as to heat-shock. Once adapted to BC, biofilm cells were better able to survive BC than were planktonic cells. The alterations in cell surface roughness, fatty acid composition, and protein expression of

the two phenotypes are in agreement with their ability for survival. There are inherent problems associated with the control of biofilms, such as the poor penetration of the antimicrobial agents, dilution effects, as well as neutralization and degradation of the agents. Thus, deeply embedded biofilm cells are the most difficult to kill using antimicrobial agents. The enhanced survival ability of BC-adapted biofilm cells would act synergistically with these other factors, thereby contributing to the persistence of biofilms even after proper sanitation. BC-adapted planktonic cells may also have a better chance to attach, multiply, and to form biofilms in BC-containing environments. This is also significant in the context of biofilm control and eradication. Thus, there is the possibility of enhanced survival and re-growth of BC-adapted biofilms relative to the untreated biofilms following lethal BC exposures.

7. GENERAL DISCUSSION

Salmonella serovar Enteritidis has emerged as one of the most exigent of all foodborne enteric pathogens in recent decades. It has been reported that the majority (~97%) of the strains of this organism are capable of forming biofilms in food processing environments (Korber et al., 1997; Solano et al., 2002; Stepanović et al., 2003), a feature of significance from the perspective of food safety and public health. Furthermore, adaptive resistance of *Salmonella* serovars to biocides routinely used in healthcare facilities and the food industry (e.g., BC, chorhexidine, and triclosan) as well as chemotherapeutic agents (e.g., erythromycin) has also been reported (Braoudaki and Hilton, 2004, 2005; Mangalappalli-Illathu and Korber, 2006), with some adapted strains also showing cross-resistance to related or unrelated chemotherapeutic agents (Langsrud et al., 2004). Therefore, research on the adaptive responses and physiology of *Salmonella* serovar Enteritidis biofilms to various environmental stressors, such as hydrodynamic conditions and antimicrobial exposure, are important.

There are reports that various physiological mechanisms operate in concert at different hierarchical levels, many times synergistically, in order to confer enhanced resistance to biofilms when challenged with various environmental stressors (Mah and O'Toole, 2001; Szomolay et al., 2005). Responses including cell surface changes, cellular morphological modification, alterations in cellular fatty acid composition, and induction of stress response pathways, all reportedly contribute to bacterial adaptive resistance to antimicrobial compounds (Sakagami et al., 1989; Braga and Ricci, 1998; Szomolay et al., 2005; Mangalappalli-Illathu and Korber, 2006). My thesis research examined the effect of altered environmental conditions, such as nutrient laminar flow velocity as well as BC treatment, on the adaptive responses of *Salmonella* serovar Enteritidis biofilms.

It was found that low-flow biofilms consisted of diffusely-arranged microcolonies which grew until merging by ~72 h; whereas, high-flow biofilms were significantly thicker and consisted of large bacterial mounds interspersed by water channels. The lectin-binding analysis of biofilm exopolymers revealed a significantly higher proportion of GalNAc in low-flow biofilms (55.2%), relative to only 1.2% in high-flow biofilms. Alternatively, the proportions of α-L-fucose and GlcNAc2–NeuNAc polymer conjugates were significantly higher in high-flow biofilms than low-flow biofilms. Despite an apparent flow velocity-based physiologic effect on biofilm structure and EPS glycoconjugate composition, no major shift in protein expression was seen between the low-flow and high-flow biofilms, and notably did not include any response involving the stress response proteins (DnaK, SodB, and Tpx). It is presumed that long-term adaptive response to a stress condition would be distinct from the response that immediately follows the first-time exposure to the condition. In the current situation, adapted biofilms may no longer require the expression of the above stress response proteins. Thus, the stage of adaptation process is suggested to be decisive in determining the response to exposure to a stress condition. There was, however, a significant difference in the protein expression patterns between planktonic and biofilm phenotypes. It is hypothesized that the biofilms altered their architecture in response to flow, thereby assuming a structure that minimized overall biofilm stress. When high-flow biofilms were exposed to the empirically-determined shear-inducing flow, biofilm thickness was reduced by approximately 70%, leaving the non-shearable region attached to the substratum. There was no significant difference in the EPS glycoconjugate composition of the two biofilm regions. However, length:width indices of the cells in non-shearable and shearable biofilm regions, as well as planktonic cells from biofilm effluent and continuous culture indicated that the cells in the shearable biofilm fraction were morphologically more similar to planktonic cells than to the cells in non-shearable biofilm region. The enhanced expression of proteins involved in cold-shock response, stress-adaptation, and broad regulatory functions (CspA, GrcA, and Hns, respectively) in the shearable region, and enhanced expression of protein involved in heat-shock response and chaperonin function (DnaK) in the non-shearable region indicated that the physiological status of cells in the two biofilm regions was distinct. The differential

cellular, morphological and physiological changes in the two biofilm regions are suggested to be adaptive responses to different stressors prevailing in the respective biofilm microenvironments.

The development of adaptive resistance following either continuous or intermittent sub-lethal exposure of biofilms to BC was examined. Biofilms adapted to BC over a 144 h period could survive a normally-lethal BC challenge and then re-grow. The exposure of untreated biofilms to the lethal BC challenge resulted in biofilm erosion and cell death. The proteins up-regulated following BC adaptation were those involved in energy metabolism (Eno and TpiA), amino acid and protein biosynthesis (DsbA, RplL, RpoZ, TrxA, Tsf, Tuf, and WrbA), nutrient-binding (FruB), adaptation (CspA), detoxification (Tpx, SodB, and a probable peroxidase), and 1,2-propanediol degradation (PduA and PduJ). A putative universal stress protein (YnaF) was also found to be up-regulated. The proteins involved in proteolysis (DegQ), cell envelope formation (RfbH), adaptation (UspA), heat-shock response (DnaK), and broad regulatory functions (Hns) were down-regulated following the adaptation. An overall increase in cellular protein biosynthesis was deduced from the significant up-regulation of ribosomal subunit proteins, translation elongation factors, and an amino acid biosynthesis protein as well as down-regulation of serine endoprotease. Cold-shock response, stress response, and detoxification are implicated in the adaptive resistance of the biofilms to BC. Subsequently, it was found that the proportion of BC-adapted biofilm cells that survived a lethal BC concentration and heat-shock at 55^{o}C was significantly higher than that of BC-adapted planktonic cells. The survival of BC-adapted planktonic and biofilm cells exposed to lethal BC concentration was significantly higher than that of their unadapted (i.e., untreated) counterparts. Overall, the surface roughness of biofilm cells was found to be significantly higher than that of planktonic cells. The fatty acid composition was significantly influenced by both phenotype (planktonic vs. biofilm) and BC treatment. The key proteins up-regulated in BC-adapted planktonic and biofilm cells were CspA, TrxA, Tsf, YjgF, and a probable peroxidase STY0440 (putative thiol-alkyl hydroperoxide reductase). Nine and 17 unique proteins were up-regulated in BC-adapted planktonic and biofilm cells, respectively. The results suggested that enhanced biofilm-specific up-regulation of 17 proteins along with the increased expression of CspA, TrxA,

Tsf, YjgF, and a probable peroxidase STY0440, as well as phenotype-specific changes in cell surface roughness and fatty acid composition operated synergistically to confer enhanced survival to BC-adapted biofilm cells in comparison to BC-adapted planktonic cells.

The experiments described herein have demonstrated that *Salmonella* serovar Enteritidis biofilms adapted to changing environmental conditions by responding at the community, cellular, and sub-cellular levels. Community level adaptation was evidenced by alterations in the biofilm architecture, biofilm thickness, total biomass and viable to nonviable biomass ratio at different biofilm depths, biofilm shearability, and biofilm EPS glycoconjugate composition. At the cellular level, adaptation functioned via changes in cellular morphology and cell surface roughness. Adaptation at the sub-cellular level included the differential regulation and expression of proteins and fatty acids following exposure to the stressors. There are reports suggesting that microorganisms continually redefine and adapt themselves at multiple levels, including molecular, cellular, and community levels (Sonea and Panisset, 1983; Sonea, 1991; Caldwell et al., 1997c; Caldwell, 2002; Caldwell and Caldwell, 2004). The findings of my research are clearly supportive of these conclusions. As suggested in these reports, the proliferation of a nested series of structures (e.g., genes proliferating within cells, cells proliferating within communities) results in a logic circuit that calculates the form and function of each structural element in the series. In the present study, exposure to stress (e.g., BC) resulted in adaptive responses, which are evolutionary responses for survival that function at the sub-cellular, cellular, and community levels in a calculative manner such that the organism could efficiently evade the deleterious effects of the stressors and survive. Moreover, all the adaptive responses may not be elicited in the event of exposure to a particular stress factor. The organism logically chooses the response which would effectively protect it from the stressor, in a calculative manner. It is presumed, in this case, that the organism elicited these mechanisms very efficiently. For example, in laminar flow conditions the organism rearranged the community (i.e., microcolonies) structurally and altered its EPS glycoconjugate composition, so that the overall biofilm stress became reduced to a minimum with the formation of water channels, thereby alleviating the requirement for a response involving the expression of

stress response proteins. Similarly, a more significant stressor, such as BC exposure, resulted in cellular (changes in cell surface roughness) and sub-cellular (changes in protein and fatty acid composition) responses, because BC exposure resulted in the erosion of biofilms. Thus, architectural adaptation may have been impossible in this particular case. It is intriguing as to why there are significant differences in the adaptive responses of planktonic and biofilm phenotypes. One possible explanation is that planktonic cells are free to swim (*Salmonella* serovar Enteritidis planktonic cells are motile) and move away from the stressor, and thus there is a relatively good chance for survival even without having a strong adaptive response. However, in the case of biofilm cells, they are attached and are relatively less motile (FliC (phase 1 flagellin) and FljB (phase 2 flagellin) are down-regulated in the biofilm phase). Thus, their "stationary lifestyle" necessitates efficient adaptive mechanisms such as changes in cell surface roughness, differential protein expression, and modified fatty acid profile, so that their survival is ensured.

Salmonella serovar Enteritidis biofilms architecturally-adapted to changing nutrient laminar flow conditions by altering their structure, thickness, as well as exopolymer glycoconjugate composition. The protein expression patterns of the biofilms were essentially similar, indicating that the distinct biofilm architectures and patterns of EPS composition exhibited under the different flow regimens was a physical consequence of flow, but did not involve major shifts in protein expression. These findings suggest that complex mechanisms are operational that result in modification of extracellular glycoconjugates that are not dependent upon large scale shifts in protein expression, or that chemical evidence of this event was simply not seen since proteomic analysis was only performed at 168 h and thus, the "adaptation event" had occurred much earlier. The findings are also supportive of the notion that biofilms are automatonic systems that respond to physical and chemical conditions such as flow rate and nutrients, with the end result being different biofilm architectures (Van Loosdrecht et al., 1997; Wimpenny and Colasanti, 1997). Biofilm protein expression patterns were significantly different from that of planktonic cells. It is suggested that the biofilms were architecturally-adapted to changes in substratum chemistry, flow velocity and thus linked to nutrient availability and waste removal, thereby reducing overall biofilm stress.

This represents an evolutionary response to life on surfaces and helps explain how *Salmonella* serovar Enteritidis cells are able to survive and persist under apparently hostile conditions imposed within environments such as healthcare facilities and the food industry. The effect of nutrient composition on biofilm architecture and physiology was not compared in this research.

The architecture of biofilms was affected by flow rate, and was hypothesized to result in the formation of physiologically-distinct zones or regions. High-flow biofilms could be sheared and separated into shearable and non-shearable regions efficiently; whereas, low-flow biofilms could not. There was no significant difference in the EPS glycoconjugate composition of the two regions, with the lectin analyzed. However, the use of a greater variety of lectins for lectin-binding analyses of the two biofilm regions might have indeed identified differences in EPS composition. It is significant that thicker biofilms harbored cells with varying phenotypes (i.e., cells with different morphology and physiology) dependant upon their location (i.e., deeper or peripheral region) in the biofilms. The cells which are located in the deeper layers of thicker biofilms are presumably in a prolonged stationary phase, and consequently unaffected by the antimicrobial (disinfectant) treatment. These cells easily become adapted to the antimicrobial compounds because of the stress response mechanisms which are already operational in these stressed cells, due to nutritional and spatial limitations. Biofilms could also harbor "persister" cells (Lewis, 2001), and these cells could also become cross-resistant to many related and unrelated antimicrobial compounds and/or chemotherapeutic agents. Persister cells are difficult to eliminate by routine antimicrobial treatment and cleaning procedures because they simply possess the ability to "persist". Moreover, biofilms are more resistant to antimicrobial treatment in terms of structure as a consequence of poor penetration, dilution effects, neutralization of the agent, etc. Along with this, the augmented adaptive responses of the biofilm cells relative to planktonic cells could undoubtedly be responsible for enhanced survival of the surface-associated cells, as demonstrated in this research.

Various molecular mechanisms have roles in the bacterial adaptive responses to antimicrobial compounds. These adaptive mechanisms act either singly or synergistically to confer resistance; among them, "slow" multiplication resulting in

"persisters", SOS response, cold-shock response, stress response, detoxification, altered permeability of the outer membranes due to changes in LPS composition, outer membrane proteins, cytoplasmic membrane proteins, fatty acid composition and content of cytoplasmic membrane, cell surface charge, hydrophobicity, active efflux of the agent etc. are believed to be significant (Karlowsky et al., 1996; Bianchi and Baneyx, 1999; Spoering and Lewis, 2001; Loughlin et al., 2002; Braoudaki and Hilton, 2005; Levin and Rozen, 2006; Mangalappalli-Illathu and Korber, 2006). It was reported that a single microorganism may have multiple, possibly interconnected, adaptive mechanisms depending on the nature of the antimicrobial agent (Campanac et al., 2002; Szomolay et al., 2005). In this study, proteins involved in degradation (carbon compounds), energy metabolism (glycolysis and pentose phosphate pathway), protein biosynthesis, cell processes (nutrient-binding), and adaptation to stress conditions became up-regulated in the biofilm cells of *Salmonella* serovar Enteritidis relative to planktonic cells. Furthermore, the proteins involved in degradation (carbon compounds), energy metabolism (glycolysis), biosynthesis (amino acids and co-factors), protein biosynthesis, cell processes (detoxification and nutrient-binding), and cold-shock response became up-regulated more substantially in the biofilm cells following BC adaptation. A schematic illustration of the metabolic pathways in operation of biofilm cells of *Salmonella* serovar Enteritidis is presented in Figure 7.1.1. It is interesting that some proteins (PduA, RpoZ, Tsf, Tuf, YcbL, and YnaF) were expressed at relatively low levels in the untreated biofilm cells; whereas, these proteins were significantly up-regulated following BC adaptation. However, the other proteins (CspA, DsbA, Eno, FruB, GntY, PduJ, RplL, SodB, TpiA, Tpx, TrxA, WrbA, YjgF, a probable peroxidase STY0440, and a putative periplasmic protein) were expressed only following BC adaptation. The significance of these two different protein expression patterns during BC adaptation of the biofilms cells is yet to be fully elucidated. Future studies involving specific gene knock-out mutant(s) would provide more information in this regard.

There was a significant difference in the expression of two proteins involved in protection against oxidative stress (Tpx and SodB) between BC-adapted planktonic and biofilm cells. Biofilm cells adapted to BC up-regulated Tpx and SodB, whereas, planktonic cells did not up-regulate these proteins. Similar differences existed in the

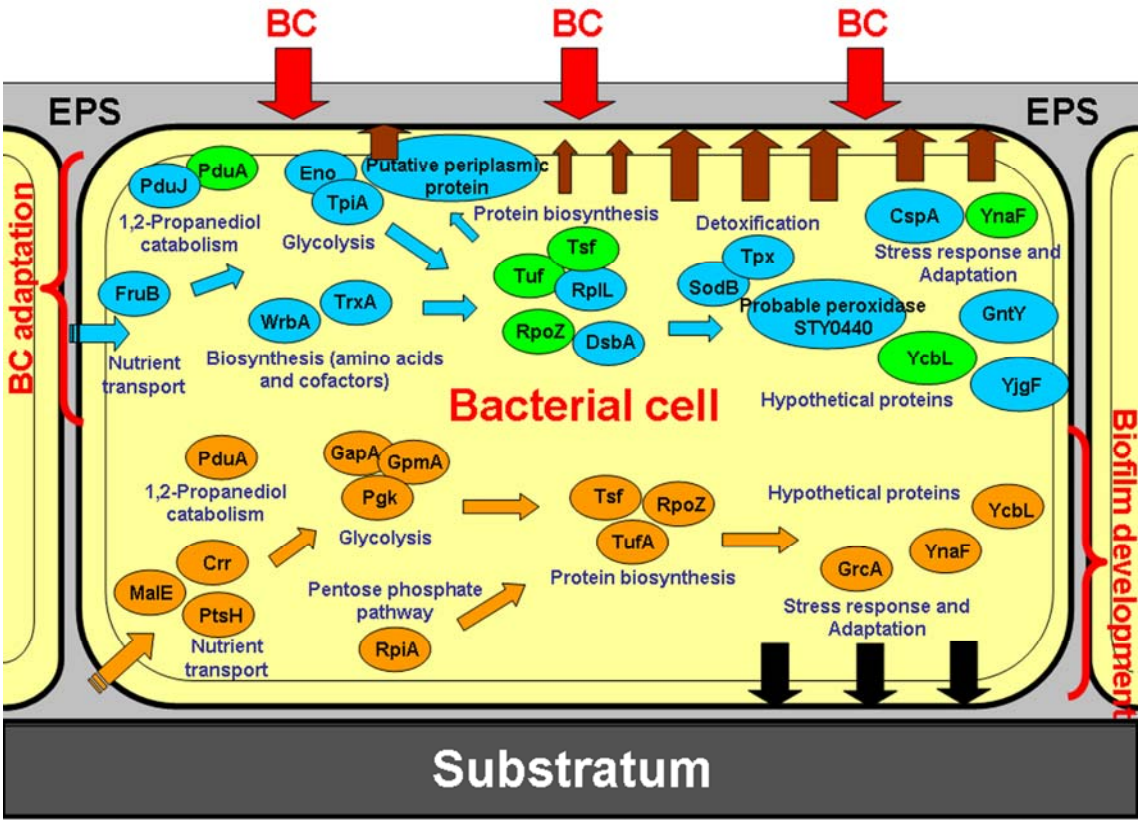


Figure 7.1.1. Schematic illustration of proteins and metabolic pathways involved in the development and BC adaptation of *Salmonella* serovar Enteritidis biofilms, as determined based on proteomic data obtained from 168 h high-flow biofilms. The proteins indicated by orange, blue, and green shades respectively, are those up-regulated during biofilm development, those only up-regulated following BC adaptation, and those primarily up-regulated during biofilm development and then significantly up-regulated following BC adaptation. The arrows within the cell, indicated by black and brown shades denote defensive response of the cell mediated through effector proteins against the stressors present in the biofilm microenvironment (e.g., nutrient depletion, waste product accumulation, and spatial limitation) and BC stress, respectively.

expression of proteins involved in degradation (PduA and PduJ), amino acid and protein biosynthesis (DsbA, RplL, RpoZ, Tuf, and WrbA), and nutrient-binding (FruB) as well as proteins with hypothetical (GntY, YnaF, and YcbL) and unknown functions (putative periplasmic protein) between BC-adapted planktonic and biofilm cells. The expression of these proteins along with increased expression of CspA, TrxA, Tsf, YjgF, and a probable peroxidase STY0440, coupled with phenotype-specific alterations in cell surface roughness and shift in fatty acid composition are suggested to have operated synergistically to confer enhanced survival to BC-adapted biofilm cells relative to BC-adapted planktonic cells.

Biofilm adaptation to environmental stressors such as antimicrobial exposure and mechanical disturbances has very practical implications in the control of biofilms found in healthcare facilities, the food industry, and even households (e.g., the kitchen sink). This study also provides an appropriate model for the development of resistance in biofilms of non-pathogenic spoilage organisms that may form in the food industry. Adapted biofilms are considerably more difficult to be removed by routine cleaning procedures, especially when the biofilms are protected by surface heterogeneities. Possible solutions clearly necessitate preventing the formation of biofilms via improving the design of equipment to avoid biofilm formation, and also by adopting multi-faceted cleaning procedures. This include judicious use of antimicrobial compounds, effective scrubbing, as well as preventing the re-attachment of the organisms after their removal by the implementation of advanced techniques, such as the use of special bacteria-repelling coatings on the surfaces of the equipments and also the use of super-high magnetic fields, ultrasound treatment, and high-pulsed electric fields to repel bacteria from the surfaces (Kumar and Anand, 1998). There is further need to improve our understanding of the key factors influencing biofilm formation and the adapted phenotype in order to develop effective control strategies in the future.

8. SUMMARY AND FUTURE DIRECTIONS

This thesis research comprised of studies that examined adaptive responses of *Salmonella* serovar Enteritidis biofilms to environmental stress conditions, such as nutrient laminar flow and BC exposure. The biofilms adapt to the stress conditions by means of community, cellular, and sub-cellular level responses. These adaptive responses help the biofilms to enhance their ability for survival in the nature. The adaptation of pathogen biofilms to various environmental stresses has practical implications in the control of biofilms, especially those formed in critical environments, such as healthcare facilities, the food industry, and households. Biofilms adapted to environmental stresses are difficult to be eliminated by routine cleaning and sanitation procedures. The application of advanced biofilm prevention strategies could be useful in the control of biofilm formation in critical locations.

Based on my thesis findings, there are some areas which require further investigations, as noted below:

1. Even though a 10-fold difference in nutrient laminar flow velocity influenced the biofilm structure and EPS glycoconjugate composition, there was no significant change in the biofilm's proteome. It would be interesting to examine how the biofilms behave with regard to its structure, EPS glycoconjugate composition, and the proteome if the difference in the flow velocity was increased by 100-fold or more. Comparisons could be made between biofilms grown under low laminar flow and turbulent flow conditions, for the most wide-ranging comparison. A more substantial difference in the flow velocity might influence biofilm thickness, biofilm shearability, the EPS glycoconjugate composition, and

possibly, the cellular morphological and physiological parameters, more perceptibly.

2. The mass transfer of nutrients in the biofilm microenvironment is dependent upon the medium flow velocity. It would be important to know how a changing nutrient concentration and nutrient composition within the biofilm microenvironment impacted the biofilm architecture and proteome. Changes in nutrient concentration/composition could be examined either alone or in conjunction with flow velocity (*see* above) to determine the influence on various biofilm structural and physiological parameters.

3. The inclusion of a larger panel of lectins for the EPS glycoconjugate analyses of shearable and non-shearable biofilm regions might identify differences in their EPS glycoconjugate composition that weren't seen in the present work. Similarly, a time-course analysis of lectin-binding might be valuable to detect the changes in exopolymer chemistry during the formation and development of the biofilms.

4. The cell surface roughness data obtained using AFM appeared to provide valuable information on cellular response to antimicrobial agents. If the roughness data could be correlated with changes in membrane fatty acid and outer membrane protein composition, this could be valuable knowledge. The effect of physical agents, such as heat/cold on cell surface roughness of BC-adapted cells, might be investigated.

5. The compositional change(s) in the EPS glycoproteins following BC adaptation of *Salmonella* serovar Enteritidis biofilms is not known. The sub-lethal BC exposure resulted in significant reduction in biofilm thickness, without being lethal to the cells. It is possible that the reduction in thickness was due to direct/indirect effect of BC on the EPS and subsequent biofilm erosion.

Therefore, the influence of BC adaptation on the changes in proportion and composition of EPS glycoproteins might be of significance.

6. The roles of the proteins, such as GntY, PduA, PduJ, YcbL, YjgF, YnaF, and the putative periplasmic protein (*see* Table 5.4.1) in the adaptive resistance of *Salmonella* serovar Enteritidis cells to BC requires more study. Knocking out the gene(s) of interest (e.g., periplasmic protein), or following their expression via RT-PCR/microarray could help identify key elements in the BC resistance pathway.

7. The "early phase" adaptive events following BC exposure and the effect of lethal BC treatment on the proteome of BC-adapted cells requires further study.

8. The role of global regulators such as RpoS and CsrA in the adaptive resistance of *Salmonella* serovar Enteritidis cells to BC may be investigated.

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